Characterisation of a novel pluripotent stem cell survival compound

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Summary

Human pluripotent stem cells (hPSC) such as human embryonic stem cells (hESC) and induced pluripotent stem cells (hiPSC) are incredibly valuable tools for investigations within a number of scientific fields including developmental biology, toxicology, pharmacology and perhaps most importantly, regenerative medicine. HPSC have an unlimited capacity for self-renewal which allows the expansion of clinically relevant cell numbers from a relatively small supply of starting material. Furthermore, hPSC are pluripotent, meaning they retain the capacity to differentiate into all the somatic cell types within the human body.

In order for the huge potential of hPSC to be realised, many hurdles must first be overcome. The most basic of these is the development of consistent and scalable culture systems that allow sufficient expansion of hPSC without the loss of the stem cell identity. Critical to this matter is the susceptibility of hPSC to apoptosis upon enzymatic disaggregation wherein approximately 80% of hPSC begin the process of apoptosis. Recent efforts to overcome this issue have focussed on the Rho associated coiled-coil kinase (ROCK) inhibitor Y27632. However there is increasing evidence that the use of Y27632 can lead to an increased risk of karyotypic instability, a decrease in proliferative capacity and a reduced capacity to differentiate in to specific cell types such as haematopoietic cell types.

The work presented within this thesis describes the characterisation of T16, a novel hPSC survival compound which did not inhibit ROCK in a previously performed *in vitro* kinase assay.

Initial experiments confirmed the capacity of T16 to support hPSC survival upon enzymatic dissociation to a comparable level to that obtained through use of Y27632, regardless of culture medium or extracellular matrix (ECM) used. Furthermore T16 was shown to support enzymatic passage of hPSC for at least 20 consecutive passages when used either transiently or continuously without any detrimental effect on stem cell marker profile, karyotypic stability or differentiation capacity.

The hyperphosphorylation of myosin light chain (MLC) has been reported to be the cause of dissociation induced apoptosis, with ROCK inhibition able to prevent this. Experimentation confirmed that T16 does not share this mechanistic target and does not prevent the hyperphosphorylation of MLC, or mediate its pro-survival effect upstream of ROCK activation via inhibition of RhoA (Ras homologous member A). Furthermore T16 does not promote survival by promoting activation of Rac1.

Further experimentation revealed that the pro-survival effect of T16 was dependent upon re-attachment to the ECM, however T16 did not increase the expression of a panel of integrins or other adhesion related proteins. Kinase inhibition studies identified a critical role for src-family kinases in T16 mediated survival of hPSC. Biochemical analysis showed that T16 did not alter the phosphorylation status of src-family kinases. Transcriptional analysis revealed Receptor for activated protein kinase C (RACK1) to be highly expressed in hPSC and subsequent experiments confirmed RACK1 as a binding partner for src-family kinases in hPSC, and that treatment with T16 alters this relationship.

The transcriptional response to dissociation was analysed via microarray analysis and identified the Pi3K and integrin signalling pathways as highly regulated pathways upon dissociation of hPSC.

In summary, the work presented within this thesis identifies T16 as a novel pro-survival molecule that mediates its effect in a RhoA/ROCK/MLC independent, src-family kinase dependent manner.

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Author's Declaration

I declare that this thesis has been written entirely by myself and is a record of work performed by myself with the exception of in vitro kinase assays, which were performed at The International Centre for Kinase Profiling, Dundee and the karyotypic analysis which was performed at the Medical Genetics department, Yorkhill Hospital, NHS Greater Glasgow & Clyde. The transcriptomics was performed in conjunction with the Glasgow Polyomics Facility. This thesis has not been submitted previously for a higher degree. The research was carried out in the Institute of Cardiovascular and Medical Sciences, University of Glasgow, under the supervision of Dr Jo Mountford.

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List of abbreviations

µg	microgram
µg/ml	microgram per millilitre
µl	microlitre
µM	micromolar
µm	micrometre
Abr	active BCL related
AFP	Alpha fetoprotein
ANOVA	Analysis of variance
APC	Allophycocyanin
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
C3	Clostridium botulinum C3 exoenzyme
CDC42	Cell division cycle 42
cDNA	Complementary DNA
CM	Conditioned medium

co-IP	Co-immunoprecipitation
CS	CellStart
C_t	Cycle threshold
DAPI	4 , 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
EB	Embryoid body
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	ethylene glycol tetraacetic acid
FA	Focal adhesion
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FCS	Foetal calf serum

FDA	Food and drug administration
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FN	Fibronectin
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDI	Rho guanine nucleotide dissociation inhibitor
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GMP	Good manufacturing practice
GTP	Guanine triphosphate
hiPSC	Human induced pluripotent stem cells
hPSC	Human pluripotent stem cells
HRP	Horse radish peroxidise
ICM	Inner cell mass
IGFR	Insulin-like growth factor receptor
ILK	Integrin linked kinase
IPTG	Isopropylthio-ß-galactoside

IVF	*in vitro* fertilisation
IκK	IκB kinase
L	Litre
M	Molar
MEF	Mouse embryonic fibroblast
mESC	Mouse embryonic stem cells
mg	milligram
mins	minutes
ml	millilitre
MLC2	Myosin light chain 2
mM	millimolar
mRNA	messenger RNA
NEAA	Non-essential amino acids
Oct4	Octamer-binding transcription factor 4
PBS	Phosphate buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PI	Propidium iodide

Pi3K	Phosphatidylinositol-3-OH kinase
PKC	Protein kinase C
qRT-PCR	Quantitative real time polymerase chain reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
RACK1	Receptor for activated C-kinase 1
RBC	Red blood cell
RFU	Relative fluorescence units
RhoA	Ras homologous member A
RIPK2	Receptor interacting protein kinase 2
RNA	Ribonucleic acid
RNAi	RNA interference
ROCK	Rho associated coiled-coil kinase
RPM	Revolutions per minute
RTK	Receptor tyrosine kinase
SCNT	Somatic cell nuclear transfer
SDS	Sodium dodecyl sulphate
Secs	Seconds
SEM	Standard error of the mean

SMA	Smooth muscle actin
SMAD	Mothers against decapentaplegic homolog
Sox2	Sry-box 2
SSEA4	Stage specific embryonic antigen 4
TBST	Tris buffered saline with tween
TF	Transcription factor
TGFβ	Transforming growth factor beta
TLDA	TaqMan Low Density Array
Tra-1-81	Tumour receptor antigen 1-81
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VN	Vitronectin
x g	Relative centrifugal force
α	alpha
β	beta

1 Introduction

1.1 Pluripotent stem cells

Human pluripotent stem cells (hPSC) such as human embryonic stem cells (hESC) and induced pluripotent stem cells (hiPSC) are incredibly valuable tools for investigations within a number of scientific fields including developmental biology, toxicology, pharmacology and perhaps most importantly, regenerative medicine. HPSC hold great appeal to scientists primarily due to two key features; their capacity for unlimited self-renewal and their pluripotency. Pluripotency refers to their ability to derive any of the 220+ cell types encompassing the three germ layers that make up the human body (mesoderm, endoderm and ectoderm) (Amit *et al*, 2000; Thomson *et al*, 1998). This means that from a single cell line, scientists can generate huge numbers of cells that can be further expanded or differentiated into the various somatic cell types.

1.1.1 Human embryonic stem cells

Embryonic stem cells have been extensively studied for a number of years, having been first isolated from mice in 1981 (Evans and Kaufman, 1981; Martin, 1981) and non-human primates in 1995 (Thomson *et al*, 1995). However, a major breakthrough occurred in 1998, when Thomson and colleagues described the isolation and characterisation of hESC for the first time. HESC were isolated from the inner cell mass (ICM) of the pre-implantation blastocyst which had been produced as a result of clinical *in vitro* fertilisation (IVF) treatments (figure 1.1). The cells of the ICM were isolated via immunosurgery during which the extraembryonic trophectoderm cells were lysed and washed away, before being cultured on irradiated mouse embryonic fibroblast (MEF) cells and allowed to further expand (Thomson *et al*, 1998). Since then there have been a large number of additional hESC lines produced using variations on this methodology. Although there is inherent heterogeneity between hESC lines (Allegrucci and Young, 2007), there are a number of criteria that all hESC must meet. These include the expression of a number of pluripotency related cell surface markers and transcription factors such as stage specific embryonic antigen 4 (SSEA4), SSEA3, tumour receptor antigen 1-81 (Tra1-81), Tra-1-60, octamer-binding transcription factor 4 (Oct4), Nanog and Sry-box 2 (Sox2). Further essential properties include an expanded capacity for proliferation (self-renewal), multi-

lineage differentiation potential and the retention of normal euploid karyotype of 46, XY/XX (Hoffman and Carpenter, 2005).

Although there are now many different hESC lines, they are all morphologically similar, growing in small colonies characterised by tight cell-cell adhesions mediated by cadherin proteins and have large nuclear to cytoplasmic ratios with very prominent nucleoli (Oh *et al*,2005).

1.1.2 Induced pluripotent stem cells

Although still considered by many to be the 'gold standard', there are a number of issues with the use of hESC not least the ethical issues relating to the destruction of embryos. Furthermore their use in regenerative medicine may be hampered given that the resulting cells would most likely be allogenic and would therefore require the subsequent and persistent use of immunosuppressive drugs (Preynat-Seauve et al, 2009). These issues were potentially circumvented in 2007 when Takahashi and colleagues reported the derivation of the first ever human induced pluripotent stem cell (hiPSC) line. This landmark and Nobel Prize winning research used a cocktail of retroviruses containing the pluripotency related transcription factors Oct4, Sox2, Klf4 or c-Myc to reprogram terminally differentiated somatic cells, specifically skin fibroblasts, to an embryonic like state (figure 1.1). Not only were these cells morphologically indistinguishable from hESC, but they had equivalent expression of pluripotency markers, similar proliferative potential and were able to generate tissues from all three germ layers. These reprogrammed cells have a number of advantages over standard hESC lines in that they have the potential to be used as personalised therapies by reprograming the patient's own cells to provide subsequent autologous treatments (Beltrão-Braga et al, 2013). Furthermore they can be used as excellent models for understanding the development of genetic diseases, with models already being generated for a range of disorders such as Parkinsons (Soldner *et al*, 2009), Huntington disease (Park *et* al, 2008) and Downs syndrome (Mou *et al*, 2012).

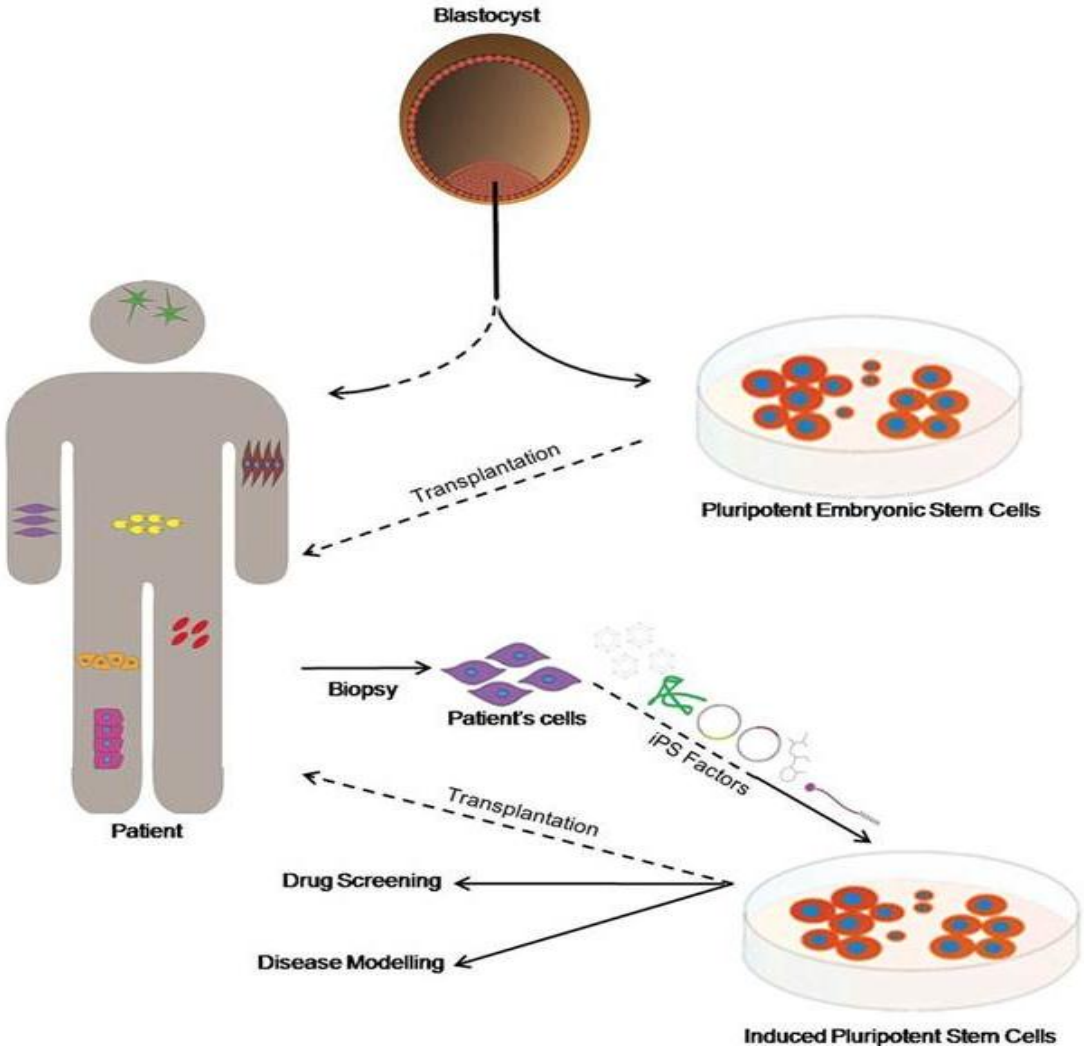


Figure 1.1. Derivation of hPSC. HESC are derived from the ICM of the pre-implantation blastocyst. HiPSC are created by the direct reprogramming of somatic cell types using key reprogramming factors such as Oct4, Sox2, Klf4 and c-Myc. Both cell types can be cultured indefinitely in vitro and can be used as potential tissues in regenerative medicine.

The use of hiPSC is not however without issue. There have been many questions raised regarding the use of viruses as a means of introducing the reprogramming factors as this introduces the risk of insertional mutagenesis. Furthermore, the use of oncogenes such as c-Myc, which is one of the key transcription factors used during reprogramming, has also caused concern (Yamanaka, 2012; Medvedev *et al*, 2010; Okano *et al*, 2013). There has been a large body of data published that details the use of non-integrating methods of reprogramming as well as variations on the factors required for efficient reprogramming. For example, Yu *et* a*l* (2007) successfully produced hiPSC without the use of c-myc, instead using a combination of Oct4, Nanog, Lin28 and Sox2. Furthermore, Okita and colleagues achieved reprogramming by using episomal plasmid vectors to introduce a number of factors and further enhanced reprogramming efficiency

and safety by suppression of p53 and the substitution of c-Myc with l-Myc (Okita et al, 2011). Similarly, Fusaki et al (2009) used the non-integrating RNA based Sendai virus coupled with the original Yamanaka factors to achieve non-integrative reprogramming.

Another interesting method of reprogramming utilises cell penetrating peptides directly linked to reprograming proteins (Yamanaka factors) which entirely circumvents the requirement of any carrier (Kim et al, 2009). A comparison of the advantages and disadvantages of hESC and hiPSC as well as the derivation methods of hiPSC can be seen in tables 1.1 and 1.2 respectively.

Cell type	Potency	Origin	Disadvantages	Advantages
hESC	Pluripotent	ICM of pre-implantation blastocyst	Ethical issues regarding source Complex tissue culture Teratoma potential Allogeneic	Pluripotency More efficient derivation
hiPSC	Pluripotent	Somatic cells via reprogramming	Poor reprogramming efficiency Use of oncogenes Teratoma potential Complex tissue culture Insertional mutagenesis	Pluripotency Autologous Disease modelling Ethical consent

Table 1.1- Comparison of hESC and hiPSC. Table showing the sources and potency of hiPSC and hESC as well as the various advantages and disadvantages associated with each.

Recent efforts to improve hiPSC efficiency and safety have focussed on the use of small molecules to induce reprogramming rather than transcription factors, as well as the use of less mature precursor cells as reprogramming targets. For example, Kim *et al* (2008) have shown that neural stem cells can be reprogrammed through the use of only Oct 4 and Klf4, or with Oct4 alone using mouse cells (Kim *et al*, 2009). It is thought that reprogramming is more effcieint in these less mature cells because they already express endogenous Sox2.

It has been reported that small molecules such as BIX-01294 and BayK8644 are able to facilitate the production of mouse iPSC when used in combination with only 2 factors; Oct4 and Klf4 (Shi *et al*, 2008). This work was further advanced by Hou *et al* (2013) who recently managed to produce mouse iPSC using a cocktail of 7 small molecules without the need for any additional reprogramming factors. Although this work provides proof of concept, reprogramming with small molecules alone has not yet been achieved using human cells, however Zhu *et al* (2010) have generated hiPSC using small molecules in combination with Oct4 alone. Further investigations into the control of hPSC behaviour will lead to a greater understanding of the mechanisms that regulate pluripotency and survival, and may elucidate alternative or more efficient reprogramming factors that might present additional targets for small molecule intervention, removing the need to use genetic reprogramming factors.

1.1.3 Somatic cell nuclear transfer

The possibility of somatic cell nuclear transfer (SCNT) as a means to reprogram terminally differentiated adult cells has been investigated since initial research performed on amphibians in the 1960s (Gurdon, 1962), however, the research is perhaps most well recognised as the technology that was used in the seminal experiments that resulted in the first cloned mammal, Dolly the sheep (Wilmut *et al*, 1997). The great hope for SCNT in humans was that the resulting reprogrammed oocytes could be fertilised and used as a means to produce personalised hESC that could be used to either study disease development, or for autologous tissue production (Yang *et al*, 2007). Despite great interest, researchers struggled to produce embryos of sufficient quality that were able to develop beyond the 8-cell stage (French *et al*, 2008). However, these problems were recently overcome by Tachibana *et al* (2013), who reported the successful derivation of hESC following SCNT. Although this development was reported very recently and will require additional investigation, it demonstrates an alternative source of hPSC that may be compared to both the standard IVF derived hESC and hiPSC in the coming years (Tachibana *et al*, 2013).

Type of vector	Method	Genomic integration	Factors	Reference
Virus	Retrovirus	Yes	Oct4, Sox2, KLF4, c-Myc	Takahashi *et al*, 2007
	Lentivirus	Yes	Oct4, Sox2, nanog, Lin28	Yu *et al,* 2007
	Adenovirus	No	Oct4, Sox2, KLF4, c-Myc	Zhou and Freed, 2009
	Sendai Virus	No	Oct4, Sox2, KLF4, c-Myc	Fusaki *et al*, 2009
DNA	Episomal Plasmid	No	Oct4, Sox2, KLF4, c-Myc	Yu et al, 2009
	Transposon	No	Oct4, Sox2, KLF4, c-Myc	Woltjen *et al*, 2009
	Minicircle	No	Oct4, Sox2, nanog, Lin28	Jia *et al*, 2010
RNA	RNA	No	Oct4, Sox2, KLF4, c-Myc	Warren *et al*, 2010
Protein	Cell permeable proteins	No	Oct4, Sox2, KLF4, c-Myc	Kim *et al* 2009

Table 1.2- Summary of hiPSC derivation methods. Overview of the various methods used for the derivation of hiPSC, including the reprogramming factors and information on genomic integration. Adapted from Okita and Yamanaka, 2011.

1.1.4 Regenerative medicine

The use of haematopoietic stem cells as an effective treatment for a number of conditions such as haematological cancers (leukaemia) has demonstrated the incredible potential of stem cells as therapeutics (Van Zant and Liang, 2012). The possibility of deriving replacement cell types for future transplantation remains one of the most exciting applications reported for hPSC. However, in order to produce viable clinical applications for hPSC a number of hurdles will need to be circumvented these include the development of efficient lineage specific differentiation protocols at good manufacturing practice (GMP) quality, the effective removal of undifferentiated cells to eliminate the risk of teratoma formation, and the development of appropriate strategies to enable delivery or to target hPSC to the required tissues (figure 1.2) (Wu and Hochedlinger, 2011). Although these are substantial issues, they are not insurmountable and a number of recent trials using hESC have been approved. The earliest approved trial was performed by Geron, and was an investigation into the use of hESC derived oligodendrocytes as a means of treating spinal trauma injuries. Although this trial was unfortunately stopped prematurely, with Geron citing financial reasons, the preliminary results were promising, with none of the patients reporting any adverse side effects to the treatment (Strauss, 2010; Ichim *et al*, 2011). Perhaps the most promising outcome from this venture was that although not without issue, the trial did reach the rigorous safety standards required by the FDA (Food and Drug Administration) for approval in the USA.

More recently, Schwartz *et al* (2012) started clinical trials that aimed to use hESC derived retinal pigmented epithelial cells as a means of treating blindness induced by macular degeneration. The directed differentiation protocol used resulted in 99% pure population of retinal pigmented epithelial cells, which successfully engrafted with the host. Reassuringly, these implanted cells showed no signs of uncontrolled proliferation, tumorigenicity or rejection after 4months. In addition to this, although difficult to accurately assess, there were signs of improved vision in a number of patients (Scwartz *et al*, 2012). Although promising, the long term effect of such treatments must still be determined, and thorough follow up will be performed on patients from both trials.

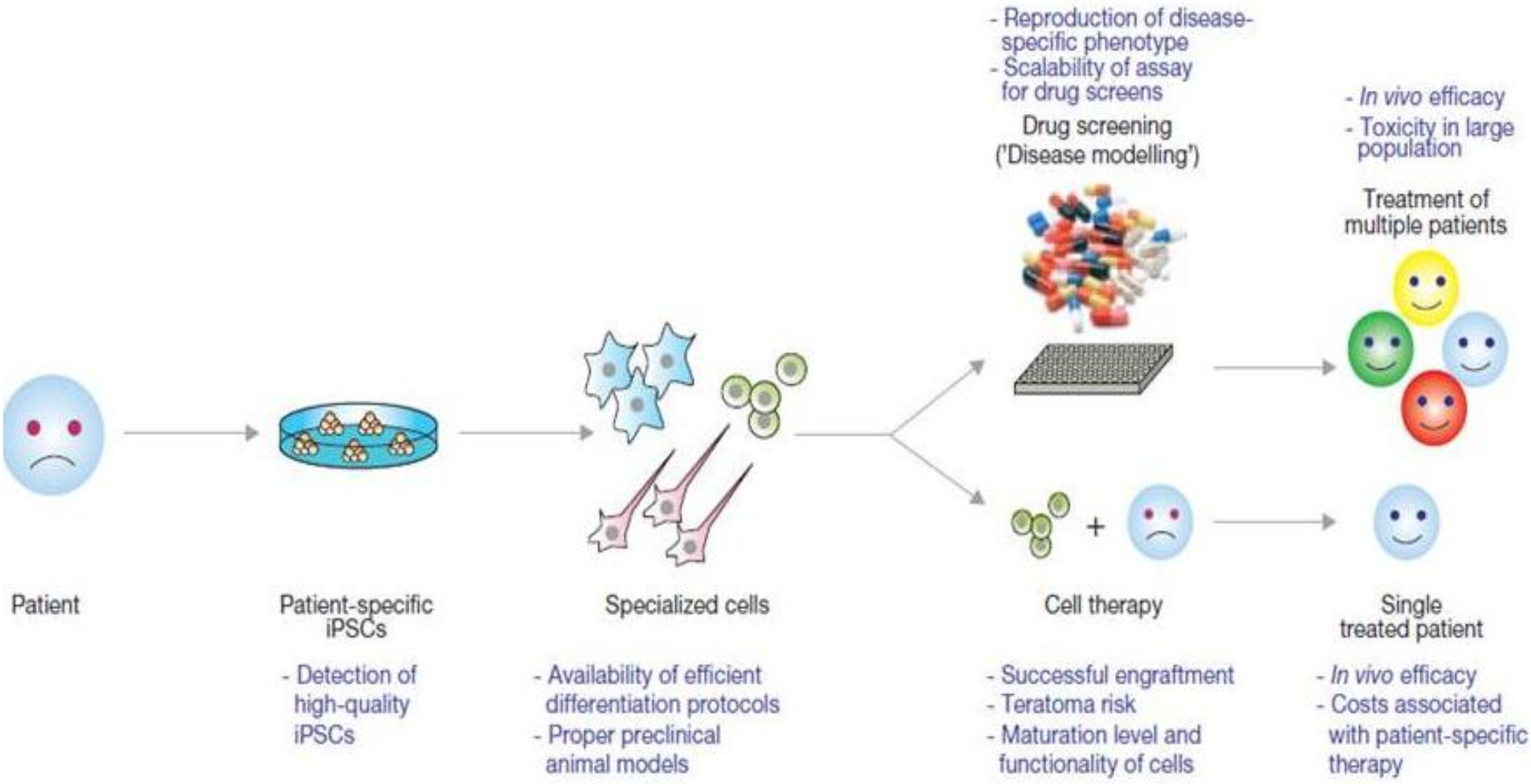


Figure 1.2- Work flow and associated problems for regenerative medicine. The use of hiPSC as a source of autologous cells for regenerative medicine would be a multi-step process, with each step presenting a number of scientific problems such as the production and isolation of high quality hiPSC, efficient differentiation and successful engraftment. Image adapted form Wu and Hochedlinger, 2011.

1.1.5 Culture of hPSC

In order for the huge potential of hPSC to be realised, many challenges must be faced; the first and most basic of these is the consistent culture of hPSC. During normal embryological development *in vivo*, pluripotent cells are present only transiently before they become committed towards precursor and mature somatic cell types of all three germ layers (Adachi and Niwa, 2013). Control of this process is extremely complicated and not yet fully understood, although it is certainly reliant on cell-cell contact and soluble mediators. Consequently, maintaining hPSC in a pluripotent state *in vitro* involves the manipulation of a number of signalling pathways.

Historically, culture of hPSC has been heavily reliant upon the use of inactivated feeder cells such as mouse embryonic fibroblasts (MEFS) to support adhesion and maintain pluripotency (Thomson et al, 1998) (figure 1.3). However, exposure of hPSC to animal products or cells raises substantial concerns including a risk of transfering non-human pathogens and also introduces a source of potential variation into the system. In an attempt to overcome these issues, various strategies have been developed such as the use of human fibroblast feeder cells for propagation (Ellerstrom et al, 2006), or more commonly, the utilisation of feeder-free culture conditions (an example of feeder-free hPSC colony can be seen in figure 1.3). As hPSC do not grow directly on standard tissue culture plastics, feeder-free culture utilises extracellular matrix (ECM) proteins such as fibronectin (FN), vitronectin (VN) or most commonly, Matrigel, to facilitate hPSC attachment in combination with MEF-conditioned medium (CM) supplemented with basic fibroblast growth factor (bFGF) to support maintenance of pluripotency (using feeder cells to condition culture medium as described in section 2.2.2.1). However, CM and ECM proteins such as Matrigel are poorly characterised and are often derived using animal sources. Matrigel for example is isolated from Engelbreth-Holm-Swarm mouse sarcoma cells and has a variety of growth factors integrated within (Hughes *et al*, 2010). This leads to a high degree of batch to batch variability that introduces inconsistences into experimental work flows that can make culture of hPSC variable, confounding the analysis of results. As a consequence of this, there has been a drive towards fully defined, xeno-free culture systems that use recombinant ECM proteins or protein fragments rather than feeder cells (Hoffman and Carpenter, 2005; Beers

et al, 2012). There have also been attempts to circumvent the requirement of ECM proteins by modifying the surface chemistry of tissue culture plates or through the use of synthetic substrates (Jin *et al*, 2012; Klim *et al*, 2010).

The drive towards fully defined culture systems will be essential if hPSC are to achieve their huge potential as clinical tools for regenerative medicine. At the heart of this are the culture media used to propagate hPSC, which must be produced at good manufacturing practice (GMP) grade (Ausubel *et al*, 2012). As previously stated, hPSC were historically cultured in conditioned medium supplemented with bFGF to support pluripotency. However the undefined nature of CM will not be acceptable if the cells are to be used clinically. In an attempt to avoid this issue, a number of groups have developed more defined media types including basic compositions such as that described by Furue *et al* (2008) who used a combination of bFGF, insulin, transferrin, albumin and heparin to support feeder-free growth. However, a recent study undertaken by The International Stem Cell Initiative Consortium), performed a thorough investigation of 8 commonly used hPSC culture mediums and found that only 2 media types were able to support successful maintenance for at least 10 passages in each of the hPSC lines tested to a comparable level of the feeder dependent control culture system (Akopian *et al*, 2010). The culture mediums in question were those with the most complex requirement for growth factor supplementation i.e. StemPro and mTeSR. However these culture mediums are still not fully defined; mTeSR is still reliant on the use of human serum albumin (Ludwig *et al*, 2006) and StemPro is routinely supplemented with BSA (Wang *et al*, 2007).

Another problem with hPSC culture media, especially those which are complex in nature or include serum albumin, is the inherent batch to batch variability associated with the various components. Akopian *et al* (2010) observed inconsistencies between laboratories who independently acquired media components, which resulted in considerable differences in the performance of the same media types. This is a substantial problem when it is considered that mTeSR for example, requires the addition of 18 factors to a DMEM based basal media which already consists of 52 components (Chen *et al*, 2011).

The dependence of many hPSC culture media on serum was recently addressed by a group who described a complex relationship between many of the media components (Chen *et al*, 2011). Chen and colleagues reported that in the absence of serum, other media additives such as ß-mercaptoethanol became more toxic to the hPSC. Using a combinatorial approach this group produced a completely defined, xeno and serum free culture medium that required only 7 additions to the basal DMEM/F12 culture medium; this media is now commercially available under the name of Essential 8. Essential 8 medium requires the addition of insulin, selenium, transferrin, l-ascorbic acid, bFGF, TGF-ß and $NaHCO_3$ (to adjust the final pH of the media) and can be used for routine culture as well as differentiation of hPSC (Chen *et al*, 2011) and is compatable with GMP-grade production.

The culture of hPSC is further complicated by their susceptibility to apoptosis upon enzymatic dissociation. Upon dissociation to single cell or small cellular aggregates, approximately 80% of hPSC begin the process of apoptosis. This issue can be overcome by using partial dissociation methods or by mechanically passaging cells using cutting tools (images of this can be seen in figure 1.3). This however is only suitable for routine passage at small scale in open vessels and requires skilled staff. This sensitivity to disaggregation poses a more significant problem in regards to cell expansion and also for genetic manipulation of cells (Watanabe *et al*, 2007). In order to produce the large number of cells required for study or indeed clinically applicable cell quantities, this issue must be overcome. This matter will be discussed in more detail in section 1.3.

Figure 1.3- HPSC cell culture pictures. Images taken using phase microscopy at x10 magnification showing hPSC (H1 hESC) colonies growing on MEF (left), a feeder-free hPSC colony (centre) and confluent hPSC that have been mechanically cut into small clumps using a cutting tool prior to passage (right). Scale bar represents 100µm.

1.1.6 Self-renewal

As previously stated, one of the important characteristics of hPSC is their capacity for unlimited proliferation and self-renewal. All stem cells regardless of potency are able to replicate indefinitely (Morrison and Kimble, 2006), however, the proliferative capacity of adult stem cells *in vitro* is significantly decreased, with cells undergoing more asymmetric divisions, which can limit the expansion of these cells and therefore their usefulness in regenerative medicine (Siddappa *et al*, 2007).

HPSC on the other hand have been reported to have been propagated continuously for at least 2 years whilst avoiding replicative senescence (Zeng and Rao, 2007). HPSC are able to replace the ends of the telomeres normally lost during cellular divisions through the enzymatic activity of telomerase, which helps them evade the effects of replicative senescence (Thomson *et al*, 1998). The control of self-renewal is also linked to and regulated by the same mechanisms that regulate pluripotency.

1.2 Maintenance of pluripotency

Although great strides have been made in recent years, the mechanisms that underlie the maintenance of pluripotency and early differentiation events remain poorly understood. Much of the recent research into this area has focussed on the key transcription factors (TF) associated with pluripotency. Transcription factors are protein or protein complexes that are able bind to promoter regions of genes and act to either activate or repress gene activation by controlling the activity of RNA polymerase (Herdegen and Leah, 1998). There are a number of key transcriptions factors that have been found to be essential for the maintenance of pluripotency in hPSC, of which, Oct4, Nanog and Sox2 are considered to be at the core of the transcriptional regulation of pluripotency. Most of the early research performed on these TF's was performed in mouse embryonic stem cells (mESC) and subsequently found to be broadly similar in hESC. However, as might be expected, there are also significant differences between the different species.

1.2.1 Oct4

Oct4 is a POU domain transcription factor that is sometimes referred to as POUF51 or OCT3/4. It is a very well conserved TF that has been shown to be present in the ICM of the blastocyst of all mammalian embryos, and loss of Oct4 expression the cells within the ICM results in loss of pluripotency (Nichols *et al*, 1998). Many studies have been carried out to determine the function of Oct4, most of which have used RNA interference (RNAi) to reduce (knockdown) Oct4 expression. Studies such as that performed by Babaie *et al* (2007) have shown that upon loss of Oct4 expression, hPSC lose other markers of stemness and begin to change morphologically within as little as 24hours accompanied by a concomitant increase in differentiation markers such as cytokeratin 18 and BMP2 (Babaie *et al*, 2007; Hay *et al*, *2004;* Matin *et al*, 2004). In addition to this, aberrant expression of Oct4 has been reported to drive hPSC to differentiate towards both endoderm (Hay *et al*, 2004) and mesoderm (Zeineddine et al, 2006) cell types.

Full transcriptome analyses of hPSC have shown the presence of the Oct4 consensus binding site in the promoter regions of over 600 known coding genes, including many of those that have been linked to pluripotency (Boyer et al, 2005). It is particularly interesting that the Oct4 gene itself is a target of the Oct4 transcription factor, indicating that the expression of Oct4 may be self-regulated in feedback and feedforward loops. Although Oct4 directly targeted 600 genes, over 1100 genes were shown to have altered expression in response to Oct4 knockdown, demonstrating the contribution to indirect regulation of genes by Oct4 (Barbaie et al, 2007). Critically, Oct4 not only target genes involved in promoting pluripotency, but also targets genes and pathways involved in triggering development and therefore differentiation (Figure 1.4). Of the 1100 genes with altered gene expression due to Oct4 knockdown, 705 of these are up regulated rather than down regulated. This suggests that Oct4 not only acts to up regulate key pluripotency genes, but also suppresses the expression of developmentally important genes.

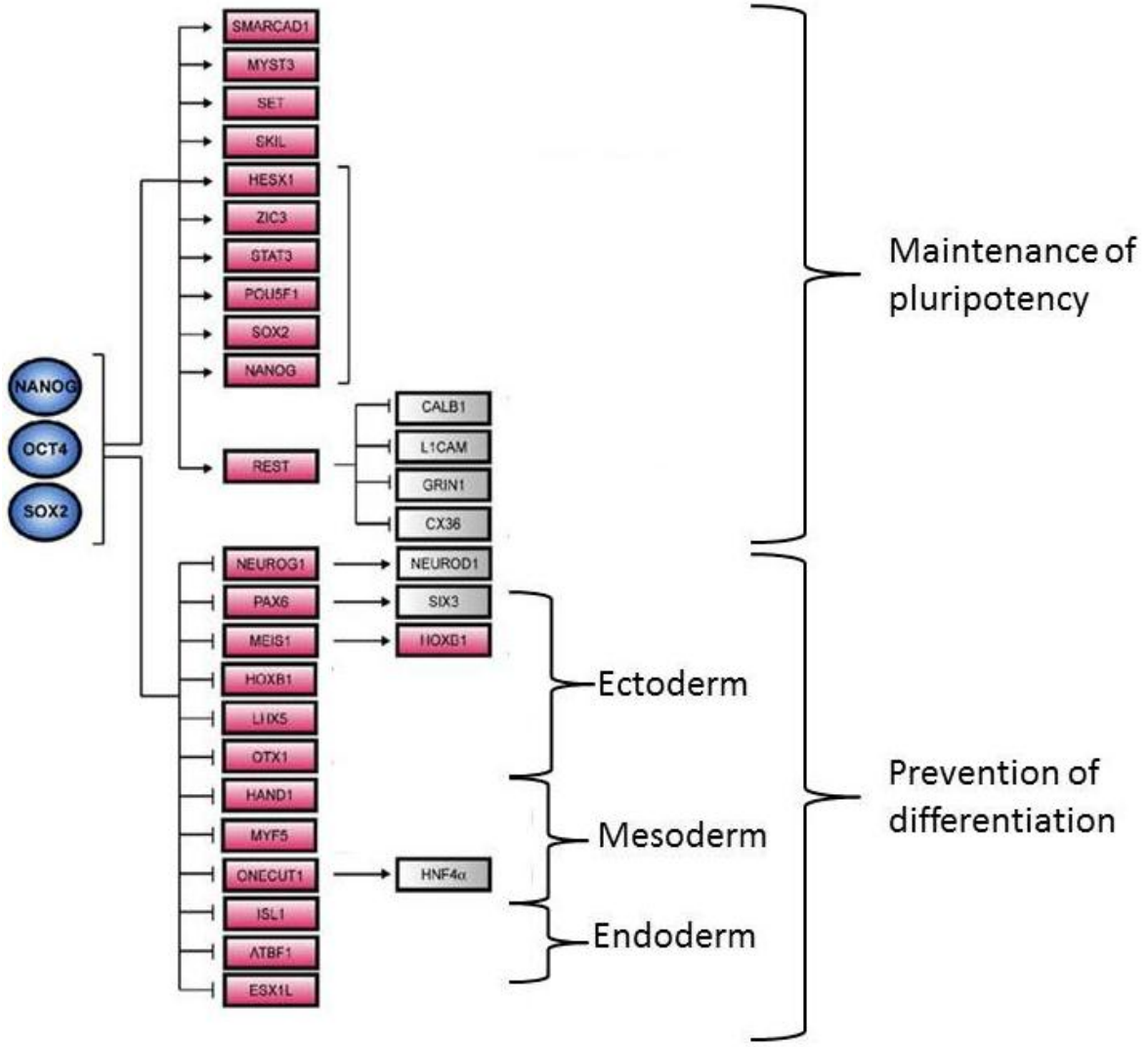


Figure 1.4- Effect of pluripotency related transcription factors on gene expression. Schematic showing that the key transcription of Oct4, Nanog and Sox2 factors not only promote the expression of pluripotency related genes, but inhibit the expression of differentiation associated genes. This also shows the presence of feedback loops as the core transcription factors all target each other. Adapted from Boyer *et al,* 2005.

1.2.2 Nanog

Nanog is a homeodomain TF that has also been heavily associated with the maintenance of hPSC pluripotency. Again, loss of Nanog expression is associated with loss of pluripotency, with hPSC differentiating towards extra-embryonic lineages (trophectoderm). In a study carried out by Hyslop et al (2005), reduced expression of Nanog resulted in reduction of other markers of pluripotency as well as increasing levels of extra-embryonic endoderm markers such as GATA4 and GATA6, within 4 days of reduced Nanog expression.

Further studies have also implicated Nanog in a series of feedback and feedforward loops, increasing the complexity of transcriptional regulation. For

example, the transcription factor smad2/3 (Mothers against decapentaplegic homolog 2/3), a downstream effector of the activin/nodal signalling pathway, has been shown to drive Nanog expression. Nanog thereafter negatively regulates smad 2/3 which prevents the differentiation of hPSC towards endoderm (Vallier et al, 2009; Darr *et al*, 2006).

Transcriptome analysis of Nanog has shown that there are >1600 genes which can be activated or supressed by binding of Nanog, which again covers those reinforcing pluripotency associated signalling as well as those involved in suppression of differentiation, a number of which overlap with those targeted by Oct4 (figure 1.5) (Barbaie et al, 2007).

Nanog has been linked closely to the maintenance of the pluripotent ground state in all mammalian cells. In particular, work performed on mESC has shown that Nanog regulation may be the key to both maintaining pluripotency and initiating differentiation; with Nanog low cells being more likely to differentiation and Nanog high cells being less likely to differentiate (Nichols and Smith, 2009; Wray *et al*, 2010).

1.2.3 Sox2

The third of the three core transcription factors involved in maintenance of pluripotency is Sox2, an HMG domain containing TF. As with Oct4 and Nanog, Sox2 is highly expressed in hPSC and is quickly downregulated upon differentiation. Sox2 has long been recognised as an important factor in hPSC pluripotency, however until recently, its function remained relatively unknown.

A number of recent studies have suggested that a key role of Sox2 is to suppress the differentiation of hPSC into trophectoderm and endoderm. Adachi *et al* (2010) have shown that the loss of Sox2 leads to a change in morphology and a switch in surface antigens from the hPSC marker SSEA4 to the differentiation marker SSEA1. Interestingly, a similar result was seen when Sox2 was overexpressed, indicating that the expression level of this TF has to be well regulated to maintain pluripotency, and furthermore, that it may have a role in differentiation pathways. Although expression of Oct4 and Nanog are reportedly specific to hPSC, the expression of Sox2 has also been detected and shown to

have a critical role in the regulation of neuronal precursor cells (Cimadamore *et al*, 2013; Johnston *et al*, 2013; Thiel, 2013).

Again as with the previous TFs, Sox2 binding sites were seen to be present in a large number of genes. Sox2 was linked to the regulation of 1271 genes, which was more than Oct4 but less than Nanog (figure 1.5) (Barbaie *et al*, 2007).

1.2.3.1 Transcription factor interactions

During their full analysis of the human hPSC transcriptome, Boyer *et al* (2005) highlighted a key relationship between the three master TFs. This work showed that although the three TFs had many distinct targets, there were a great number of target genes shared between them (Figure 1.5A). There has previously been a suggested relationship between Oct4 and Sox2 indicating that these factors dimerise to allow activation or suppression of some target genes (Ferraris *et al*, 2011). Findings showing that approximately 50% of the promoter regions occupied by Oct4 were also occupied by Sox2 suggest that this role is also conserved in hPSC. Furthermore, a huge 90% of promoter regions bound by Oct4/Sox2 were also occupied by Nanog. The authors have suggested that the proximity of these promoter regions as well as the fact that all three co-occupy 353 separate genes in hPSC is strong evidence that the three factors work together to regulate large proportions of key pluripotency associated genes (figure 1.5A)(Boyer *et al*, 2005).

Another significant point of note is that the target genes of these TFs include the Oct4, Sox2 and Nanog genes themselves suggesting that they are also able to regulate each other and indeed themselves (figure 1.5B). Taking this into consideration, it is not at all surprising to see that upon down regulation of one of these factors, the others are usually subsequently down regulated.

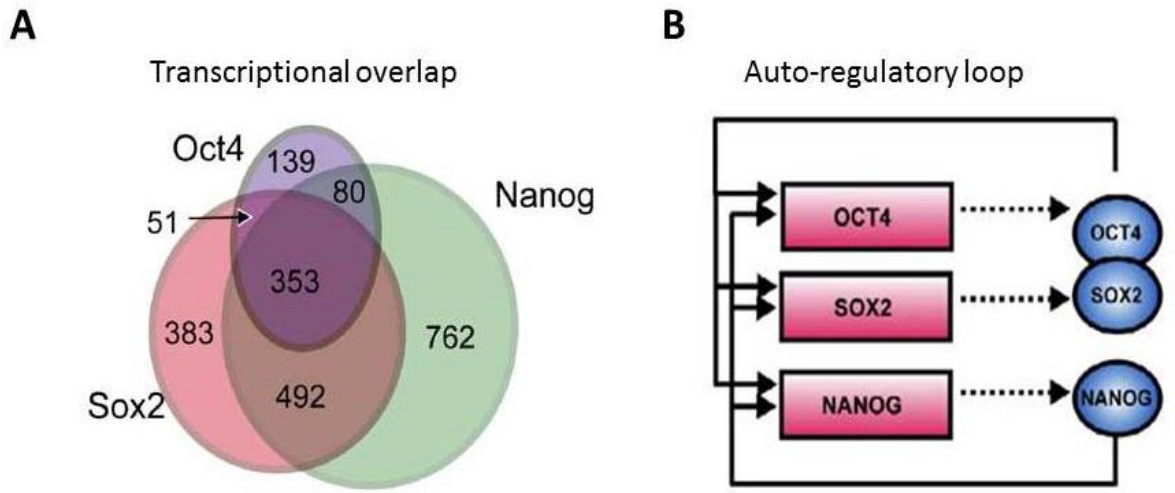


Figure 1.5- Core transcription factor interactions. Venn diagram showing the number of genes regulated by each of the key transcription factors associated with pluripotency. Each TF has unique targets as well as a large degree of common targets (A). Schematic of the auto-regulatory mechanism of Oct4, Nanog and Sox2 (B). Modified from Boyer *et al* (2005).

1.2.4 Extrisic pathways regulating pluripotency

As previously stated, there are a number of pathways that have been linked to the maintenance of pluripotency. The pathways discussed here are a mixture of those supporting pluripotency through positive regulation of key hPSC genes, and those that support differentiation by suppressing pathways integral to maintenance of pluripotency.

1.2.4.1 Fibroblast growth factor signalling

Fibroblast growth factors, or FGF's, are a family of growth factors comprised of 22 known members. These growth factors facilitate their function by binding and activating a group of receptor tyrosine kinases (RTKs), FGF receptors (FGFR). At present, there are 4 known FGFR (FGFR1-4), all of which have been shown to be expressed in hPSC (Ding *et al*, 2010). Activation of FGF signalling has for a long time been implicated as a key component in the maintenance of pluripotency, so much so that in almost all culture conditions, basic FGF (bFGF/FGF-2) is added to support pluripotency (Greber *et al*, 2010; Kang *et al*, 2005).

Due to the apparently indispensable nature of FGF signalling in hPSC, there have been many studies into its possible roles, however, the mechanisms through

which bFGF elicits these positive effect remain an issue of debate, with many studies claiming it to have different roles.

One suggested role for FGF signalling is that it acts to suppress signalling caused by bone morphogenetic protein 4 (BMP4). Introduction of BMP4 to culture conditions that would normally support undifferentiated growth of hPSC results in a change in hPSC morphology, loss of pluripotency markers and an increase in trophoblast and primitive endoderm markers characteristic of differentiation (Xu *et al*, 2005). However, it is known that even those culture conditions able to support undifferentiated growth of hPSC retain low levels of BMP's, which are largely attributed to the serum replacement products used in cell culture mediums or by autocrine production by the hPSC (Chase and Firpo, 2007; Lifantseva *et al*, 2013). BMP signalling results in activation of the TGFβ pathway via smads1/5/8, which upon phosphorylation, are able to translocate to the nucleus where they can directly influence gene expression and trigger differentiation pathways. Addition of high levels of bFGF, or a combination of bFGF and the BMP antagonist noggin, to hPSC culture systems has however been shown to ameliorate this deleterious effect of BMP. It has been suggested that bFGF may achieve this regulatory affect by inhibiting smad1 phosphorylation, or blocking the phosphorylated form from functioning within the nucleus and thereby preventing the activation of pathways associated with differentiation (Xu *et al*, 2005).

Another suggested route of action for FGF signalling is through regulation of the MEK/ERK pathway. The MEK/ERK pathway is a known downstream target of FGF signalling in a number of somatic cells types, but has also been shown to be highly active in hPSC (Kang *et al,* 2006). A recent study by Ding *et* al (2010) indicated that as early as 15minutes after bFGF stimulation of previously FGF starved cells an upregulation of phosphorylated ERK within the hPSC could be measured in response to the bFGF. The importance of MEK/ERK signalling in the maintenance of pluripotency has also been tested by targeting MEK with chemical inhibitors as well as using RNA interference. Within 3days of MEK inhibition in pluripotent culture conditions, there was a morphological change in the hPSC, and after a further 2days, transcriptional changes were evident. These changes included a reduction in pluripotency markers TRA1-60, TRA1-81, Oct4

and Nanog as well as an increase in markers of trophectoderm and primitive endoderm (Li *et al*, 2007). This supports previous work showing that FGF signalling is important in preventing the differentiation of hPSC into trophoblast and primitive ectoderm lineages (Xu *et al*, 2005).

In addition to these previously mentioned roles, Bendall *et al* (2007) have suggested another more controversial role for FGF signalling. In this study, the authors took advantage of techniques that allow separation of cells from a heterogeneous hPSC population into separate populations for subsequent analysis. Using fluorescence activated cell sorting (FACS), they found a discreet population of fibroblast like cells that were produced within cultures by the hPSC. These cells were lacking in OCT4 and SSEA3 expression whereas the colony forming hPSC were not. What was striking about this finding was that there was also a difference in the cell surface receptors being expressed by these two cell types. In contrast to previous studies showing high FGF receptor expression in hPSC, Bendall and colleagues suggested that the hPSC derived fibroblast cells within the cultures had the majority of these receptors and that hPSC were instead rich in insulin-like growth factor receptor (IGFR1). The suggested role for bFGF here was to help maintain a supportive niche within which it triggered the release of IGF2 (and TGFß) from the hPSC derived fibroblasts, which then activated IGFR1 to support hPSC growth (Bendall *et al,* 2007).

In addition to showing the potential importance of this regulatory niche that forms within hPSC cultures, Bendall *et al* also showed the importance of IGF signalling by using exogenous IGF2 (the agonist of IGFR1) to support hPSC in the absence of bFGF. Activation of the IGFR1 receptor is thought to subsequently activate, by means of phosphorylation, the phosphatidylinositol-3-OH kinase (Pi3K) pathway which has been shown to be an essential for the maintenance of hPSC by activation of smads 2/3 and the subsequent activation of Nanog (Wi *et al*, 2007; Wang *et al,* 2009).

1.2.4.2 Transforming Growth Factor Beta

The transforming growth factor beta (TGFß) superfamily of proteins is a well-represented group not only in hPSC, but also throughout the body in a number of somatic cell types. Their suggested roles include regulation of proliferation,

differentiation, migration and they are also involved in apoptotic pathways (reviewed by Valdimarsdottir and Mummery, 2005). The TGFß family encompasses a number of key pathways involved in regulation of pluripotency, such as activin/nodal, BMP and Wnt signalling.

The activin/nodal pathways have garnered great interest in recent years, and their role in pluripotency is well accepted. In a manner similar to that seen when the key TFs Oct4, Nanog and Sox2 are inhibited, knockdown of the activin/nodal pathway results in differentiation of hPSC. Activin and nodal share a number of key features, such as having the same type 1 and type 2 receptors and both utilise smad2 and smad3 as downstream effectors and regulators of transcription. Upon activation, the type 2 receptor phosphorylates the type 1 receptor, which in turn causes the phosphorylation of intracellular smad2 and smad3 (Xu *et al*, 2008). Phosphorylated smad2/3 forms a complex with co-smad4 which is then translocated to the nucleus where, as a known TF, it functions to activate relevant pluripotency associated genes (Vallier *et al,* 2009). Immunofluorescent microscopy has shown the presence of smad2/3 in the nucleus of cells expressing OCT4 and SSEA3, and the subsequent loss of this nuclear expression upon differentiation (Vallier *et al*, 2005).

Although it is clear from this that the activin/nodal signalling pathway is involved in the maintenance of hPSC pluripotency, the downstream mechanisms and indeed target genes are largely unknown. However, the bridge between extrinsic signalling pathways and intrinsic TFs has recently been alluded to by Vallier and colleagues when they showed that Nanog can both regulate and be regulated by smad2/3 (Vallier *et al,* 2009). The observation that the loss of activin/nodal signalling leads to a sudden downregulation of Nanog, as well as the identification of consensus binding sights for smad2/3 within the Nanog promoter region is strong evidence of a direct relationship between extrinsic and intrinsic components of pluripotency (Xu *et al*, 2008; Vallier *et al, 2005; Vallier* et al*, 2009*).

There has also been a suggestion of a cooperative interaction between activin/nodal signalling and FGF signalling. When nodal and bFGF were used in combination, there was higher expression of pluripotency marker SSEA4 in hPSC than when either of these receptor agonists were used alone. The importance of

the activin/nodal signalling pathway has been confirmed through use of the specific activin/nodal receptor inhibitor SB431542. Upon inhibition, even high doses of bFGF were unable to inhibit hPSC differentiation, demonstrating that both the FGF and activin/nodal pathways are crucial (Vallier *et al*, 2009).

As previously stated BMPs in hPSC have been shown to have a negative impact on pluripotency. BMPs such as BMP2/4/7 act similarly to activin/nodal signalling, activating similar receptors, but instead of activating smads 2/3, they activate smads 1/5/8. Once phosphorylated, smad 1/5/8 also bind to co-smad4 and translocate to the nucleus where rather than support Nanog expression, they inhibit Nanog. These smad 1/5/8 targets are predicted to be genes involved in differentiation pathways, including BMP2, Sox17 and FOXA2 (Pera and Tam, 2010; Teo *et al*, 2012). There has also been a suggestion that some of the beneficial effect of culturing hPSC on MEF could be due to the release of TGF signalling pathway agonists and antagonists such as nodal and GREM1 (Gremlin 1) respectively which form regulatory loops within the cultures (Pera and Tam, 2010).

1.2.5 Wnt signalling

Another extrinsic signalling pathway that has been associated with pluripotency in hPSC is the canonical Wnt pathway. There have been contradicting reports of Wnt signalling being involved in both the maintenance of pluripotency and induction of differentiation. For example, there have been suggestions that bFGF mediated regulation of the canonical Wnt pathway can lead to the upregulation of important hPSC transcription factors. Ding *et al* (2010) reported that bFGF regulates downstream targets of Wnt signalling in the absence of the Wnt ligand Wnt3a. Their suggested model for this proposes that the bFGF activated Pi3K pathway can mimic Wnt signalling by phosphorylation of GSK3, resulting in the release of ß-catenin and subsequent nuclear translocation. Once present in the nucleus, ß-catenin, as a transcription factor, can activate Wnt related genes.

An overview of the extrinsic pathways involved in maintaining the pluripotent identity of hPSC can be seen in figure 1.6. It is clear that maintenance of pluripotency and the suppression of differentiation are complex and dynamic

processes resulting from the tight regulation of multiple intrinsic and extrinsic signals.

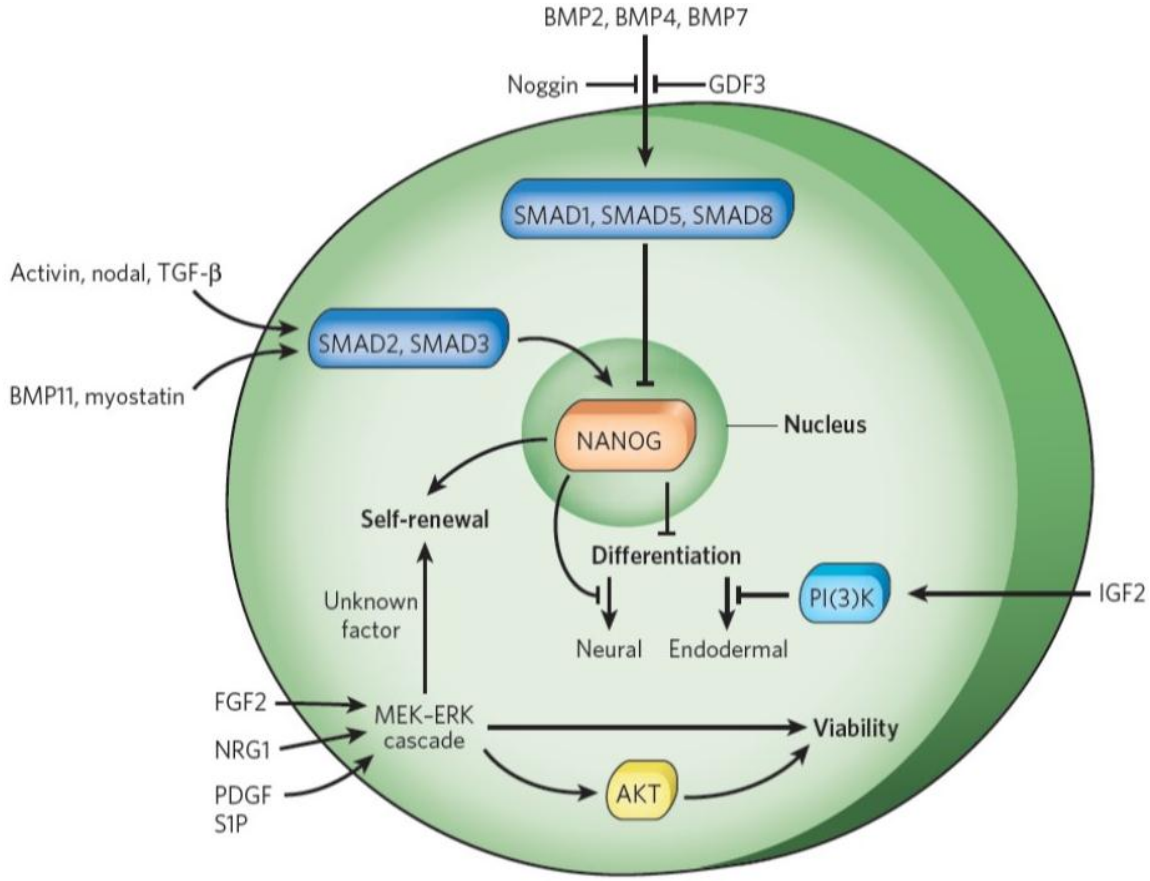


Figure 1.6- Extrinsic pathways involved in hPSC maintenance. Cell surface receptor ligands such as activin and nodal are able to trigger the translocation of smad 2 and smad 3 to the nucleus where they can drive the expression of pluripotency related genes such as Nanog. Similarly, BMPs can activate smads 1/5/8 to stimulate the activation of differentiation associated genes. This can be effectively blocked by antagonists such as Noggin. Pi3K and MEK/ERK signalling can be activated in response to FGF and IGF2 which can reinforce pluripotency related signals.

1.2.6 Epigenetic regulation of hPSC

Epigenetics refers to the regulation of gene expression and the resulting phenotypes independent of any modification to the gene sequence and explains why cells such as cardiomyocytes and neurones have such distinct morphology and functions despite containing the same basic DNA sequence (Capell and Berger, 2013). Epigenetic status is largely dependent upon histone modifications and changes in DNA methylation which can result in changes in chromatin structure. By changing chromatin structure, epigenetic modifications can

effectively control access to the regulation machinery of genes such as promoter regions. The epigenetic status of hPSC has been investigated at length and shown to be vastly different to that of their differentiated progeny (Watanabe *et al*, 2013; Han and Yoon *et al*, 2012). HPSC have been shown to have increased levels of bivalently marked domains than terminally differentiated somatic cells or multipotent precursor cells (Meissner *et al*, 2008; Mekkelsen *et al*, 2007). Additionally, the promoter regions of critical regulators of pluripotency such as Nanog and Oct4 are shown be more highly methylated in differentiated cells when compared to pluripotent cells and are therefore inactive (Maherali *et al*, 2007; Han and Yoon, 2012).

Given the role of epigenetics in regulating gene expression, it is not surprising that this has become an area of great interest when considering hiPSC derivation and differentiation. The field of epigenetics has become synonymous with the visual metaphor of Waddingtons epigenetic landscape, wherein he compares cell commitment to marbles rolling downhill (Goldberg et al, 2007). However groups have now likened the reprogramming of somatic cell types towards pluripotency as pushing the marble slowly back up a mountain (Watanabe *et al*, 2013; Hochedlinger and Plath, 2009).

Epigenetic regulation is currently an area of very active research, however, it is not directly relevant to the work described in this thesis, so whilst its importance cannot be ignored it will not be considered in detail herein.

1.3 Apoptosis in hPSC

The maintenance and expansion of hPSC is further complicated by their susceptibility to apoptosis during routine culture and passage. Although apoptosis occurs in all cell types, it has been reported that cells of the early embryo including those of the ICM from which hESC are derived, are particularly sensitive to apoptosis. It has been reported that upwards of 30% of hPSC apoptose spontaneously under standard tissue culture conditions (Qin *et al*, 2007). Of greater concern is that upon dissociation to single cell or small cellular aggregates, approximately 80% of hPSC begin the process of apoptosis. A recent study by Madden *et al* (2011) has shown that hPSC express increased levels of pro apoptotic members of the Bcl2 family of proteins when compared to either

cancer cells or terminally differentiated cells. Furthermore, hPSC have also been shown to have constitutively active Bak, another pro-apoptotic member of the Bcl2 family suggesting that hPSC may be primed for rapid apoptosis upon exposure to cellular stress (Dumitru *et al*, 2012). This sensitivity to dissociation poses significant problems with regards to basic enzymatic passage of hPSC as well as expansion to clinically relevant cell numbers. In addition, this severely limits the use of techniques that require single cells such as clonal selection of genetically manipulated cells or cell sorting via FACS (Watanbe *et al*, 2007; Pyle *et al*, 2006).

1.3.1 Intrinsic and extrinsic apoptotic pathways

Apoptosis is an evolutionary conserved form of programmed cell death that is critical for normal tissue homeostasis and is a means through which the body can remove excess or damaged cells. Apoptosis can be triggered by either intrinsic or extrinsic signalling pathways which lead to the activation of a group of cysteine proteases known as caspases (cysteine aspartic proteases). Activation of caspases results in the cleavage of a large number of cell substrates such as protein kinases and adhesion molecules, which leads to biochemical and morphological change, nuclear condensation and fragmentations and ultimately results in death and formation of apoptotic bodies (Nicholson, 1999; Coleman and Olson, 2002).

The extrinsic pathway, sometimes referred to as the receptor pathway, is triggered by activation of cell surface 'death receptors' such as Fas (CD95) receptor and is largely mediated by caspase 8 dependent activation of effector caspases such as caspase 3.

The intrinsic or mitochondrial pathway is activated in response to cellular stress such as DNA damage, and results in the release of apoptotic activators such as cytochrome c. This again results in the eventual activation of caspase 3, but is largely mediated by upstream caspase 9 activation rather than caspase 8.

Although the intrinsic and extrinsic pathways are activated and induce apoptosis via independent mechanisms, there is a certain degree of crossover between the pathways. An example of this is the activation of the intrinsic pathway via

caspase 8 mediated cleavage of Bid, resulting in Bid translocation to the mitochondria and subsequent release of cytochrome c (Fulda and Debatin, 2006). Schematic representation of the apoptotic pathways can be seen in figure 1.7.

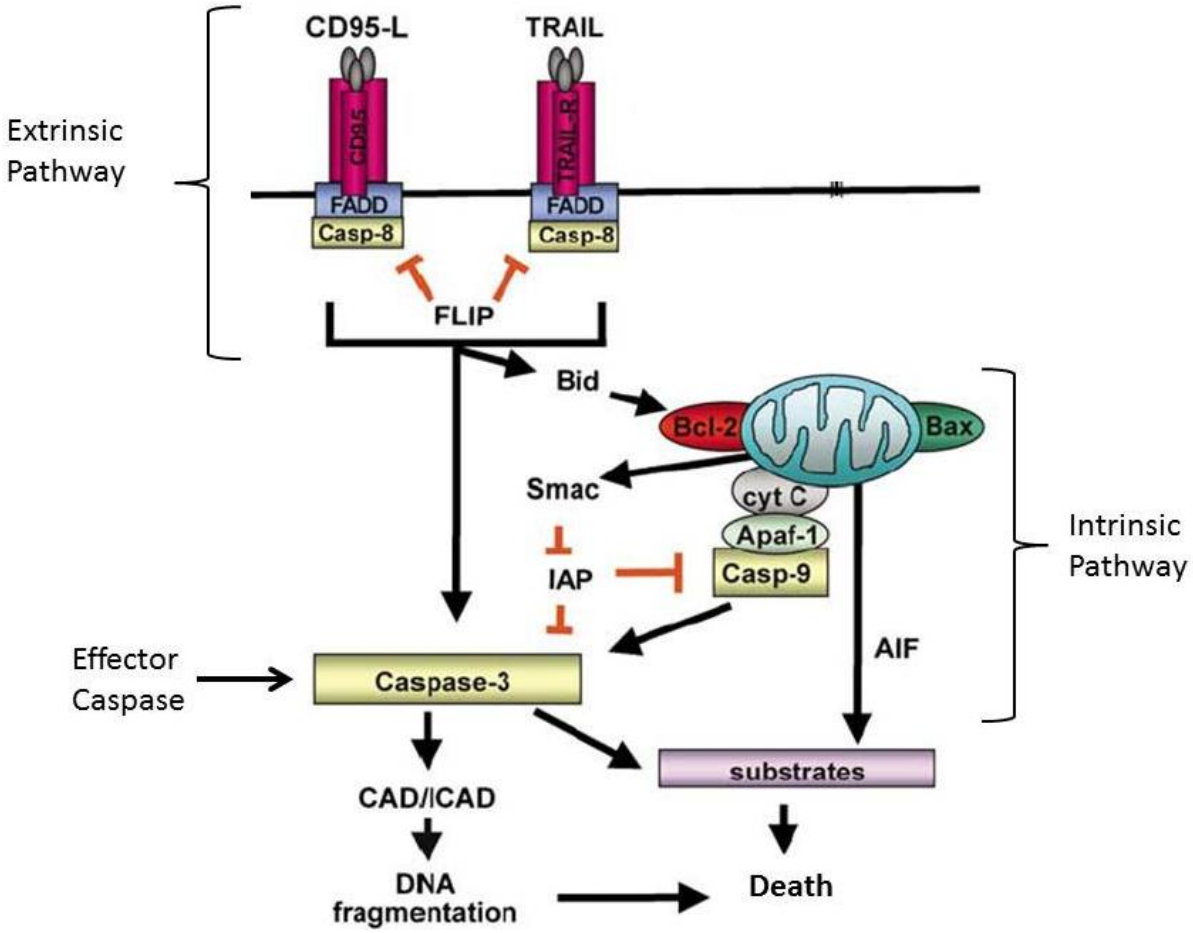


Figure 1.7- Intrinsic and extrinsic pathways in apoptosis. Schematic representation of both the extrinsic and intrinsic pathways of the process of apoptosis. The extrinsic pathway is activated by cell surface receptors and activates caspase 3 via cleavage by caspase 8. The intrinsic pathway is activated by release of cytochrome c from the mitochondria which results in activation of caspase 3 in a caspase 9 dependent manner. Adapted from Fulda and Debatin, 2006.

1.3.1.1 Anoikis

Anoikis is a specialised form of apoptosis triggered by loss of cell to matrix adherence. Anoikis, suitably named from the Greek word for 'homelessness' is a physiologically important process which prevents detached cells from migrating and proliferating uncontrollably. The physiological relevance of anoikis can be seen with cancerous cells as one of the hallmarks of cancer is the acquisition of

anchorage independent growth and suppression of anoikis (Gilmore, 2005) which also permit metastatic migration and growth.

Anoikis has been shown to facilitate apoptosis via both the intrinsic and extrinsic pathways and that the pro-apoptotic proteins Bim, Bid and Bad play a critical role. Under adherent conditions, Bim is located within a multi-protein complex, however, upon loss of integrin signalling Bim is released from the complex and translocates to the mitochondria where it can inactivate the pro-apoptotic protein Bcl-XL. Additionally, upon loss of integrin signalling there is a decrease in Pi3K dependent phosphorylation (and therefore inactivation) of Bim and Bad, which result in an augmented apoptotic response (Chiarugi and Giannoni, 2008; Cheng *et al*, 2001).

Given the anchorage dependent status of hPSC, anoikis, or pathways relating to anoikis signalling have been discussed in relation to the dissociation induced apoptosis of hPSC (Watanabe *et al*, 2007; krawetz *et al*, 2009; Wang *et al*, 2007).

1.3.2 Overcoming dissociation induced apoptosis of hPSC

A major breakthrough in the field occurred when Watanabe *et al* (2007) first described that the use of a small molecule inhibitor of the Rho associated coiled-coil kinase (ROCK), Y27632, dramatically improved the survival of hESC upon enzymatic disaggregation. They found that a brief (1hr) pre-treatment and supplementation in culture medium post passage resulted in an increase in clonal efficiency from 1% to 27% and a marked decrease in apoptosis (from 75% to ~ 15%) following dissociation.

Since its discovery in 2007, Y27632 has quickly become a very well established and commonly used component of hPSC culture and has been employed for a number of purposes such as the cryopreservation and recovery of hPSC (Li et al, 2008; Martin-Ibanez et al, 2009; Claassen *et al*, 2009), suspension culture (Watanabe *et al*, 2007; Wang *et al*, 2013) gene transfer and FACS (Kurosawa, 2012).

With the discovery that Y27632 supported enzymatic passage of hPSC, the pathways regulating activation of ROCK as well as downstream targets became central to understanding the mechanistic mode of action of Y27632 and hPSC survival.

1.3.2.1 Rho GTPases

The Rho GTPases are a subset of the Ras superfamily of small GTPases with RhoA (Ras homologous member A), Rac1 (Ras-related C3 botulinum toxin substrate 1) and CDC42 (cell division cycle 42) being the most well characterised members (Parri and Chiarugi, 2010; Bishop and Hall, 2000). As with all GTPases, they act as molecular switches able to regulate a diverse range of cellular processes including differentiation, cell division, proliferation, apoptosis, adhesion and regulation of the cytoskeleton (Jaffe and Hall, 2005; Visvikis *et al*, 2010). Rho GTPases have this potential as sensitive molecular switches due to their affinity for GDP (guanine diphosphate) and GTP (guanine triphosphate) which allows them to cycle between inactive GDP bound state and active GTP bound state. The activation status is regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and Rho guanine nucleotide dissociation inhibitors (GDIs) (Cherfils and zeghouf, 2013). GEFs facilitate the activation of Rho GTPases by catalysing the exchange of GDP for GTP. Although Rho GTPases have intrinsic hydrolytic activity, this is relatively low, meaning GTP bound Rho GTPases will remain active until the GTP is hydrolysed via the activity of GAPs (Visvikis *et al*, 2010). RhoA, Rac1 and CDC42 have their own specific GEFs and GAPs of which there are >60 of each identified (Etienne-Manneville and Hall, 2002). As a further level of control, the GDIs, act as negative regulators of Rho GTPases by altering their sub-cellular localisation (Garcia-Mata *et al*, 2011). A schematic of Rho GTPase regulation can be seen in figure 1.8.

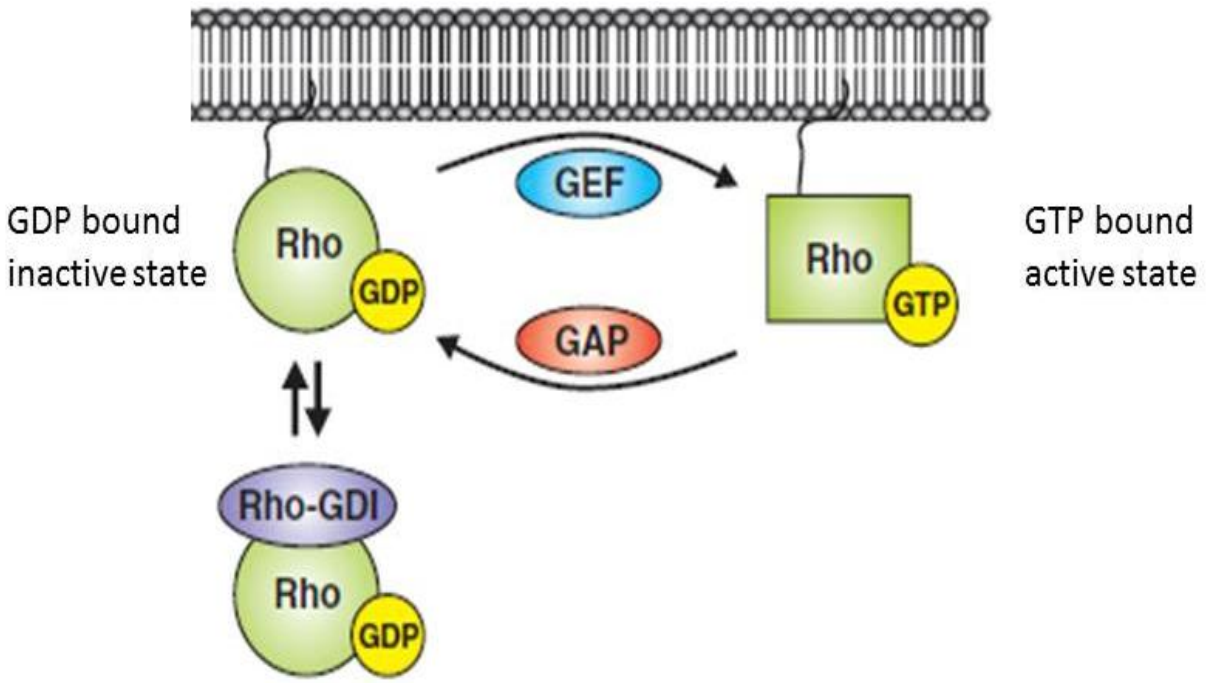


Figure 1.8- Regulation of Small GTPases. Schematic detailing the control of Rho GTPases such as RhoA, Rac1. GEFs are able to facilitate the activation of GTPases by exchanging GDP for GTP. GAPs hydrolyse GTP and therefore facilitate the transfer from an active GTP bound state to an inactive GDP bound state. GDIs act as a chaperone and localise Rho GTPases away from the plasma membrane. Adapted from Huveneers and Danen, 2009.

Signalling mediated by Rho GTPases can be initiated in response to upstream signals coming from a range a range of growth factor receptors, integrins, RTKs, cadherins or G-protein coupled receptors (Parrai and Chiarugi, 2010). When in the active GTP bound state, Rho GTPases are able to activate their various downstream effector molecules, which in turn perform various functions (as exemplified in figure 1.9). Although there is a degree of overlap with some of the downstream effectors, most have unique downstream targets. Of particular interest is ROCK, the downstream effector of RhoA and target of the hPSC survival compound Y27632. The main function of ROCK is to regulate intracellular tension via ROCK-induced actinmyosin-based contraction; it does this by its dual role in both directly phosphorylating myosin light chain 2 (MLC2) and inhibiting myosin light chain phosphatase (Iden and Gollard, 2008).

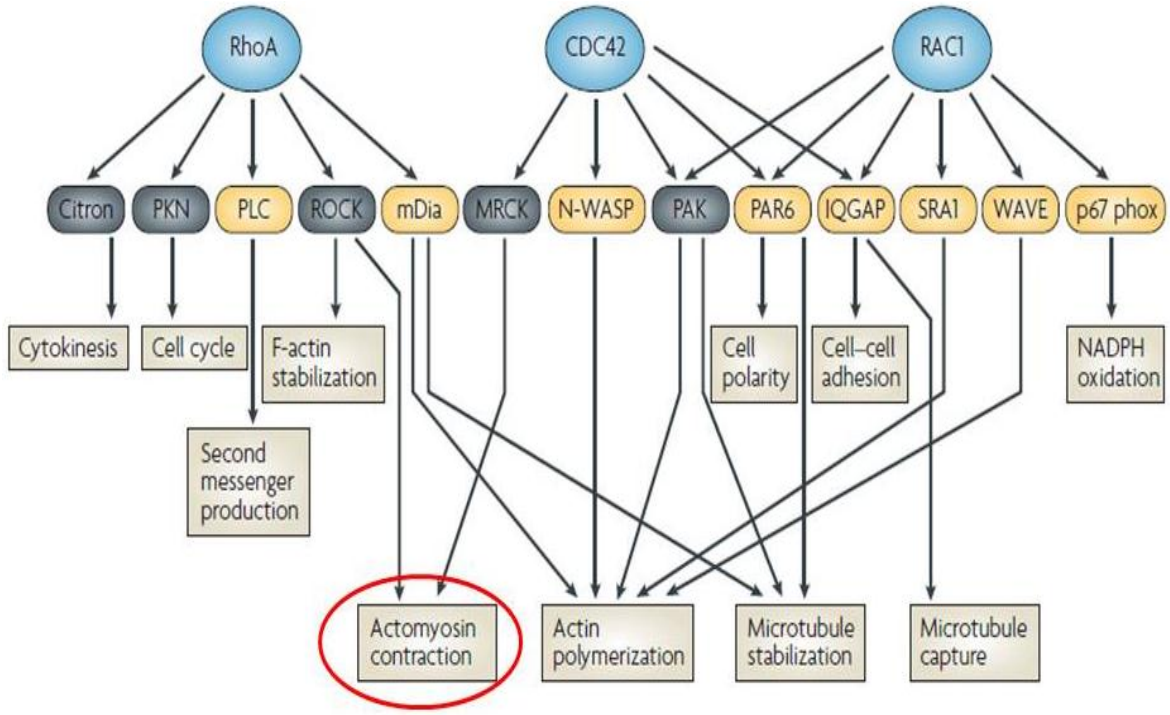


Figure 1.9- Rho GTPase effectors and functions. Schematic showing the downstream effectors of the Rho GTPases RhoA, Rac1 and CDC42 and their role within the cell. Of particular interest is the actomysosin contraction caused by ROCK. Adapted from Iden and Gollard, 2008.

Although RhoA, Rac1 and CDC42 work together to regulate this range of cellular processes, they have distinct roles and are therefore active at different times and within differing sub-cellular localisation. Of particular interest with regards to hPSC is their spatiotemporal regulation during the process of cell adhesion. At the onset of cell adhesion, there is an integrin mediated decrease in levels of GTP bound RhoA via activation of p190RhoGAP by Src kinase. This results in a loss of actinmyosin contractility and an increase in cellular protrusions such as lamellipodia and filopodia which is mediated by Rac1 activity. Initially these protrusions are rich in nascent adhesion which are either rapidly turned over or mature in to focal complexes. As the process of adhesion continues, the activity of Rac1 reduces and there is an increase in active RhoA. GTP bound RhoA mediates the activation of ROCK and the resulting actinmyosin contractility promotes the maturation of focal complexes into focal adhesions (FA) (Huveneers and Danen, 2008; Parsons *et al*, 2010; Playford and Shaller, 2004). Focal adhesion are integrin rich signalling complexes composed of actin linking proteins such as α-actinin and vinculin, adapter proteins such as paxillin, and protein kinases such as focal adhesion kinase (FAK) and Src-family kinases. This allows FA to regulate numerous downstream signalling pathways including the

small GTPases as well as pathways involved in stem cell differentiation and survival (Vicente-Manzanares *et al*, 2009). A schematic showing some of the signalling mediated by integrin activation and subsequent FAK activation is shown in figure 1.9.

As previously mentioned, RhoA and Rac1 are regulated upstream by a number of proteins, however, it has recently been shown that they have an antagonistic relationship and are also able to regulate each other. An example of this would be the ROCK mediated phosphorylation of the Rac1 GAP FilGAP which results in reduced Rac1 activity. Conversely, Rac1 can lead to a decrease in RhoA activity via inhibition of protein phosphatases which leads to a subsequent rise in p190RhoGAP activity (Huveneers and Danen, 2004). Furthermore, there have also been reports that inhibition of ROCK can have a feedback effect on the activation status of both RhoA and Rac1 and it is suggested that inhibition of ROCK leads to a Rac1 activation via Tiam1, which subsequently decreases the activation of RhoA (Tang *et al*, 2012).

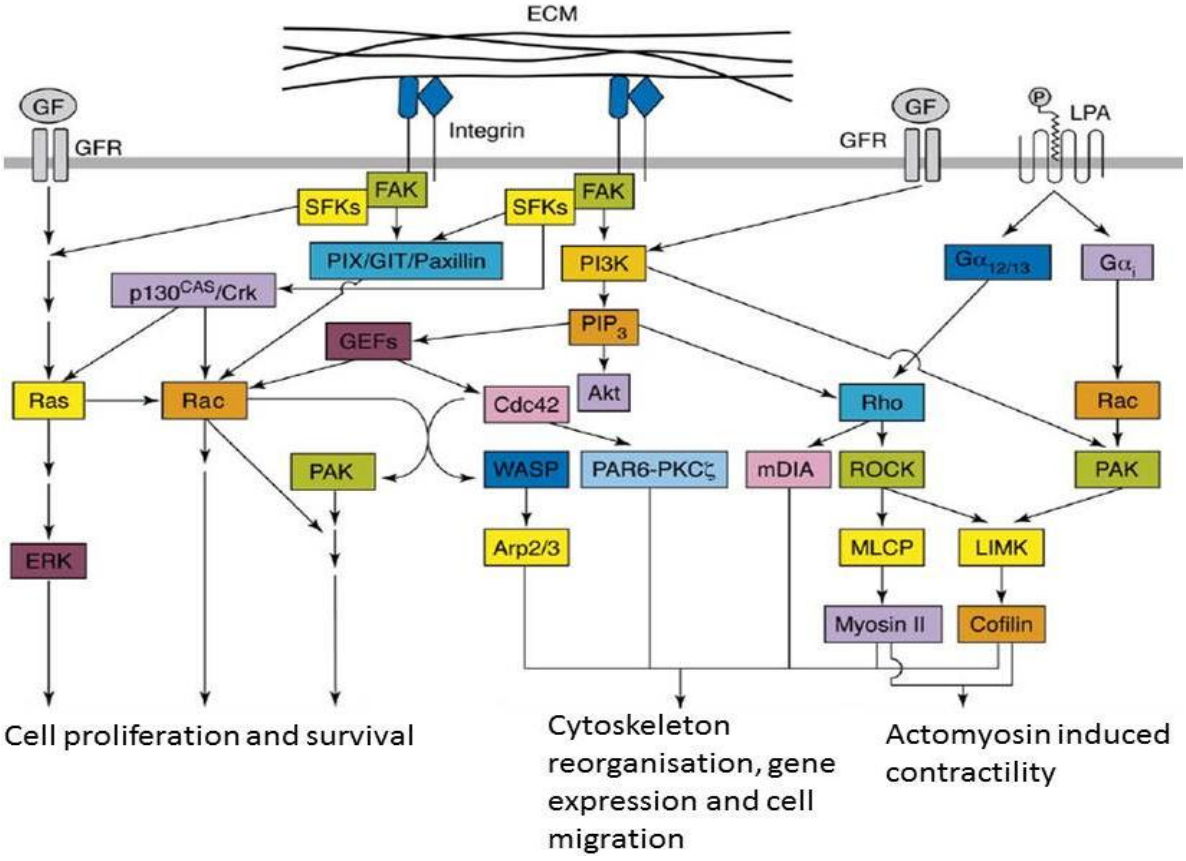


Figure 1.10- Integrin and FAK mediated signalling. Integrin signalling is activated in response to engagement with ECM components. This results in recruitment of additional proteins such as FAK and Src to the integrin cytoplasmic tail. These can activate a range of pathways with roles in diverse processes such as apoptosis, cytoskeleton rearrangements, proliferation and cell migration. Image adapted from Larsen et al, 2006.

1.3.3 Mechanistic action of Y27632

The cause of dissociation induced apoptosis of hPSC and the role that Y27632 plays in preventing it has been the subject of intense debate in recent times. This issue is complicated given that ROCK, the target of Y27632, is a downstream effector of RhoA, which has a role in a number of potentially interesting pathways such as adhesion signalling as well as apoptosis. Initially, it was thought that Y27632 promoted survival of disaggregated hPSC through an anti-apoptotic mechanism (Watanabe et al, 2007). One of the critical processes involved in apoptosis is the cleavage of ROCK by caspase 3 which results in a constitutively active form of ROCK leading to membrane blebbing and the eventual formation of apoptotic bodies (Coleman and Olson, 2002). However, it has been reported that although the inhibition of ROCK using Y27632 is able to prevent membrane blebbing and reduce formation of apoptotic bodies, it is not able to rescue cells from apoptosis. This suggests that ROCK inhibition only prevents the morphological changes associated with the regulation of the actin cytoskeleton rather than stopping cell death (Krawetz et al, 2009).

An alternative mechanism proposed for Y27632 was based on observations that Y27632 treated cells were 'stickier' than untreated cells and more difficult to dissociate from each other. Research groups therefore began to focus on the role that ROCK has on cell-cell or cell-matrix interactions. Of particular interest was the transmembrane protein E-cadherin which is involved in homotypic adhesion between cells. Reports suggested that active ROCK was able to disrupt this cell-cell signalling (Sahai and Marshall, 2002) which led groups such as Krawetz *et al* (2009) to suggest that ROCK inhibition led to increased cell-cell interaction which prevented the onset of apoptosis. Very simple experimentation by the same group provided strong support for this hypothesis when they used the Ca2+ chelator EGTA to prevent formation of Ca2+ dependent E-cadherin mediated homotypic interactions. Even in the presence of Y27632, hPSC treated with EGTA were unable to avoid apoptosis, with Y27632 treated cells having comparable levels of apoptosis to that of the control cells. However, in contrast, there have also been reports that inhibition of ROCK can lead to a decrease in cell-cell interactions in both mouse and human PSC. Interestingly, this loss of cell-cell contact was observed regardless of whether ROCK was inhibited directly with Y27632 or further upstream using the RhoA inhibitor C3 (Clostridium botulinum

C3 exoenzyme). Furthermore, the myosin inhibitor Blebbistatin also resulted in this phenotype, which suggests that the resulting inhibition of actinmyosin mediated contractility is the cause of this phenotype rather than the direct inhibition of ROCK (Harb et al, 2008).

In 2010 three independent groups published data supporting these claims and further elucidated the mechanistic action of Y27632. These groups made a direct correlation between dissociation of hPSC and hyperphosphorylation of myosin light chain 2 (MLC2), which is a downstream target of ROCK. They proposed that this hyperphosphorylation was the cause of dissociation induced apoptosis and presented data showing that treatment with either ROCK inhibitor Y27632, the RhoA inhibitor C3, or the myosin inhibitor Blebbistatin were able to circumvent this. These groups also used a combination of RNAi mediated knockdown of ROCK I and II, myosin heavy chain II, as well as the overexpression of a dominant negative form of MLC2 that cannot be phosphorylated, to further dissect the mechanism of dissociation induced apoptosis. Each of these modifications led to a decrease in the phosphorylation of MLC and a subsequent rise in hPSC survival post dissociation (Chen *et al,* 2010; Ohgushi *et al*, 2010; Walker *et al*, 2010).

Ohgushi and colleagues extended this work and also investigated signalling events upstream of ROCK and suggested that the loss of cell-cell adhesion mediated by E-cadherin lead to a sudden increase in RhoA activation in an Abr (active BCL related) dependent manner. Abr is known to have dual functionality, acting as both a RhoA GEF and a Rac1 GAP, which results in a high-Rho/low-Rac state post dissociation (figure 1.11). This work highlighted a potential antagonistic and protective role for Rac1 in hPSC, which was validated when hPSC with constitutively active Rac1 were shown to be insensitive to dissociation induced apoptosis. The authors ultimately hypothesised that the loss of cell-cell contact led to an Abl dependent shift from Rac1 activity to RhoA which leads to a sudden activation of ROCK and subsequent actinmyosin based contractility, which leads to apoptosis via the mitochondrial pathway (Figure 1.11).

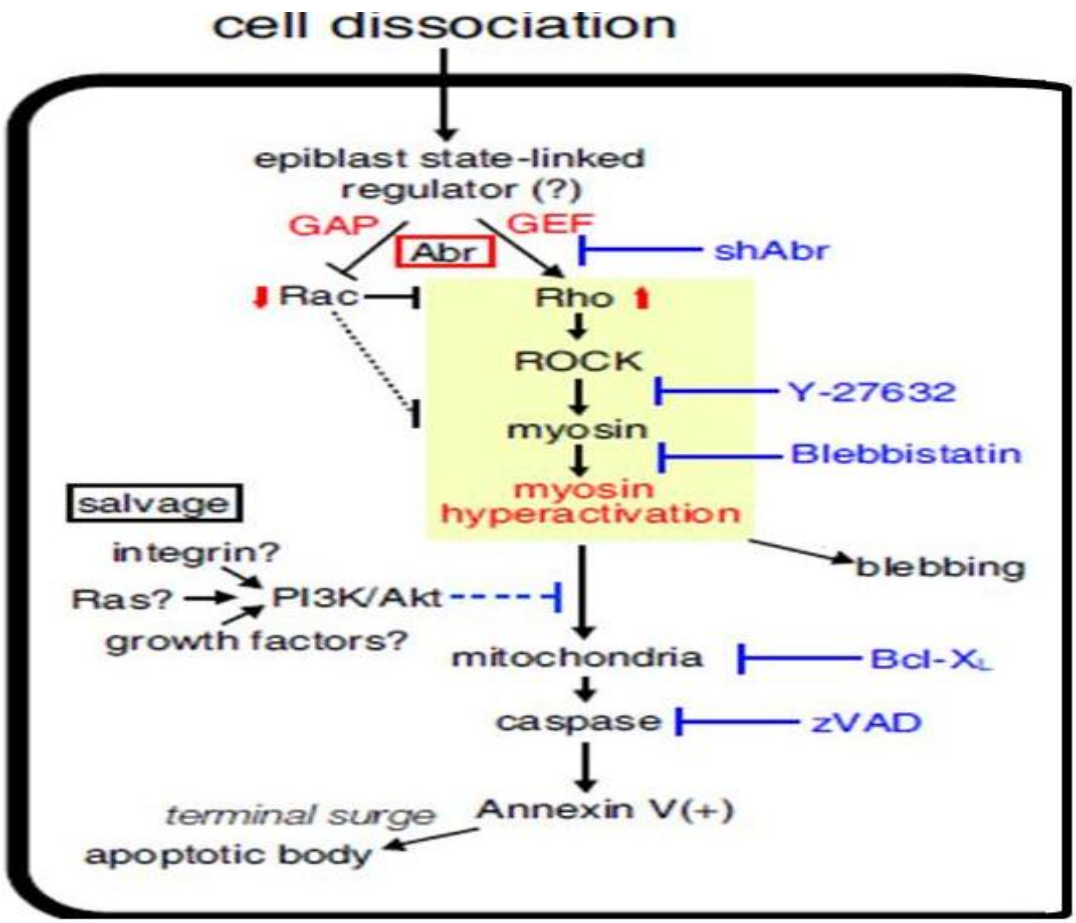


Figure 1.11- Model of dissociation induced apoptosis is hPSC. Upon dissociation or loss of cell-cell contact, hPSC undergo a sudden upregulation of RhoA mediated actinmyosin based contractility. This is Abr dependent and leads to a concurrent decrease in Rac1 activity. Apoptosis can be avoided by inhibiting this pathway at various points such as with Y27632 or Blebbistatin (blue lines). The broken blue line represents potential rescue pathways downstream of MLC phosphorylation and includes upstream regulators of Pi3K signalling pathway. Adapted from Ohgushi et al, 2010.

1.3.4 Protective pathways

Although the RhoA/ROCK/MLC pathway has been shown to be the critical pathway involved in dissociation induced apoptosis, there have been a number of additional pathways that have been implicated in protecting hPSC from apoptosis.

1.3.4.1 Integrin signalling

Integrins are a major class of transmembrane glycoprotein receptors involved in mediating adhesion between the cell and the ECM. Since their original description in 1987, integrins have become some of the most studied and best characterised proteins in vertebrates (Hynes, 1987). Structurally, integrins contain a large extracellular domain and a small intracellular domain which are

linked via a single transmembrane helix (Hynes, 2002). Currently, there are 18 known α-subunits and 8 known ß-subunits which can bind to form any of 24 known hetrodimeric integrin receptors (Brafman et al, 2013). Specific α and ß subunit pairings allow integrins to specifically bind to individual ECM components. For example, integrin α5ß1 will specifically recognise and bind to fibronectin whereas αVß5 or α6ß1 will bind to vitronectin and laminin respectively (Braam *et al*, 2008).

Integrins not only support cell-ECM adhesion, but are considered to be signalling hubs, facilitating both outside-in (Schlaepfer and Mitra, 2004) and inside-out signalling (Katagiri and Kanishi, 2012). Activation of integrins via binding to their respective ECM components, leads to conformational changes in their cytoplasmic domain resulting in the recruitment of various scaffolding proteins and signal transducing proteins and leading to subsequent signalling events that can affect not only cell adhesion but also proliferation, differentiation and cell survival. Integrins are also linked to the actin cytoskeleton via adaptor proteins such as vinculin (Johnson and Craig, 1995) and talin and are therefore major mediators of intracellular tension and cellular response to physical cues. Integrins are also able to act directly upstream of the Rho GTPases as can be seen in figure 1.10. At present, the integrin 'adhesome' is thought to comprise of around 180 proteins (Zaidel-Bar and Geiger, 2010).

As with other adherent cell types, hPSC have been shown to express a large range of both α and ß integrin sub-units. Integrins αVß3, α6, ß1, and α2ß1 have been suggested to be of most importance in regards to the adhesion of hPSC (Meng *et al*, 2010). Integrins have not only been shown to be critical in hPSC adhesion to ECM components, but have also been shown to activate pathways associated with prevention of apoptosis including the Pi3K/Akt signalling pathway.

1.3.4.2 Pi3K/Akt and FGF signalling pathways in hPSC survival

Pi3K signalling can be activated by growth factor receptors as well as by interaction between cell surface receptors such as integrins with ECM components. Upon activation, Pi3K phosphorylates Akt proteins such as Akt1, Akt2 and Akt3. Once phosphorylated, Akt proteins are able to interact with a

large array of downstream targets such as the direct regulator of apoptosis Bad (pro-apoptotic Bcl2 family member) as well as regulators of the apoptotic program such as IκK (IκB kinase) and MDM2 (mouse double minute 2 homolog) (Downward, 2004).

Pi3K signalling has also been shown to be important in hPSC and activation has been reported to increase cell adhesion and decrease apoptosis in response to stress (Eiselleova *et al*, 2009). Furthermore, expression of a constitutively active Akt mutant has been able to at least partially prevent dissociation induced apoptosis of hPSC (Ohgushi *et al*, 2010) suggesting that the RhoA/ROCK/MLC pathway might not be the sole determinant of hPSC death and survival on dissociation.

In addition to its critical role in the maintenance of pluripotency in hPSC, FGF mediated signalling has also been reported to play a significant role in the regulation of apoptosis. It has been suggested that ligand activation of the FGFR mediated by bFGF results in activation of Akt and Erk signalling which decreases caspase activation in a BIM dependent manner (Eiselleova *et al*, 2009; Wang *et al*, 2009). This mechanism of action has also been suggested to play a role in preventing anoikis of hPSC upon detachment.

1.3.4.3 Anoikis resistance

Another potential mechanism through which hPSC could prevent dissociation induced apoptosis is through resistance to mechanisms involved in anoikis. It has been previously shown that increased activity of FAK or integrin linked kinase (ILK) can protect cells from apoptosis even upon loss of ECM adhesion. This is likely due to downstream activation of Pi3K/Akt signalling as discussed above (Chiarugi and Giannoni, 2008).

Atypical regulation of epidermal growth factor (EGF) signalling can also lead to suppression of anoikis. The EGF receptor (EGFR) is normally stimulated by EGF and can lead to activation of pro-survival pathways such as the Pi3K/Akt pathway, but there have also been reports of ligand independent activation of EGFR in an integrin and Src-family kinase dependent manner. Upon integrin activation, the cytoplasmic tail can contribute to a complex containing Src and

p130Cas which can tyrosine phosphorylate the EGFR at numerous sites. The downstream signalling resulting from this can lead to phosphorylation and subsequent degradation of pro-apoptotic proteins such as Bim. During normal growth, this pathway would not be activated upon loss of integrin/ECM contact, however there have been reports that this can be activated independent of integrin activation during incidences of cell stress resulting in the release of reactive oxygen species (ROS), which can subsequently activate Src (Moro *et al*, 2002; Giannoni *et al*, 2008).

1.3.5 Stem cell state and apoptosis

Although functionally equivalent, there are major differences between hESC and mESC, such as their morphology, cell surface markers and growth factor requirements. Most interesting however, is the observation that mESC are not sensitive to enzymatic dissociation in the same way that hESC are. Although initially attributed to interspecies differences, recent evidence suggests that this sensitivity to dissociation is actually dependent on developmental stage rather than species dependent. It is now widely accepted that there are two distinct pluripotent cell types; the ICM or 'naïve' cells and the epiblast or 'primed' type cells which are developmentally more mature and are isolated from the post implantation embryo (Ohgushi and Sasai, 2011; Hanna I *et al*, 2010; Buecker *et al*, 2010). The comparison between mouse epiblast derived pluripotent cells (mEpiSC) and hPSC showed that not only do mEpiSC have similar colony morphology, surface markers and growth factor requirements, they are also sensitive to dissociation. In contrast, human epiblast like stem cells (hEpiSC) derived by nuclear reprogramming, are more comparable to mESC and are not sensitive to dissociation (see table 1.3 for a summary) (Buecker *et al*, 2010).

Pluripotent cell type	Developmental stage	Sensitivity to dissociation
mESC	Naive	Not sensitive
hESC/hiPSC	Primed	Sensitive
mEpiSC	Primed	Sensitive
hEpiSC	Naive	Not sensitive

Table 1.3- Developmental stage and sensitivity to apoptosis. Summary showing the susceptibility of developmentally discrete pluripotent cell types in response to dissociation.

1.4 Problems with ROCK inhibition

The use of ROCK inhibitors has been incredibly useful, however there have been recent concerns raised over the use of Y27632. The first of which is that Y27632 and other established ROCK inhibitors such as HA-1077 are not very specific and have been shown to inhibit a number of additional kinases with similar potency (Andrews *et al*, 2010; Davies *et al*, 2000). In addition to this, the small GTPases are a ubiquitously expressed protein family involved in a diverse range of biological processes including differentiation, cell division, apoptosis, adhesion and cytoskeleton regulation (figure 1.9) (Jaffe and Hall, 2005; Visvikis *et al*, 2010; Gerecht *et al*, 2007). Interference with such a wide range of processes has the potential to impact the balance of numerous signalling pathways and the downstream effects of Y27632 exposure in hPSC has not been investigated extensively (Couture, 2010). Zweigerdt *et al* (2011) have recently developed a culture system capable of expanding hPSC in suspension culture, however they were unable to add Y27632 after day 1 of culture due to the reduced proliferation which had been observed by other groups (Zweigerdt *et al*, 2011; Singh *et al*, 2010). In addition to this negative effect on proliferation, the use of Y27632 has been associated with an increased incidence of chromosomal abnormalities (aneuploidy) (Riento and Ridley, 2003; Liu *et al*, 2012).

Another major problem associated with the use of Y27632 is due to the critical role ROCK plays during hPSC differentiation. Our group and others are interested in the potential use of hPSC as an alternative source of red blood cells (RBC) for

transfusion, however, even transient use of Y27632 has resulted in decreased proliferative capacity (unpublished data, Mountford group) and reduced production of haematopoietic precursor cells (Yung *et al*, 2011). In support of this, Y27632 has also been shown to have a negative impact on the proliferative potential of adult CD34+ haematopoietic precursor cells (Bueno *et al*, 2010). Similarly, Y27632 has also been shown to impact the differentiation towards other cell types such as neuronal and endothelial lineages (Sivasubramaniyan et al, 2010; Sivasubramaiyan et al, 2009; Boissart et al, 2013; Joo et al, 2012).

As research into hPSC develops, further issues with Y27632 may yet be uncovered. However, despite these reported problems, there continue to be reports of a number of novel applications particularly those designed for the large scale expansion and culture of hPSC and their derivatives for therapeutic use, that are dependent upon the use of hPSC survival compounds such as Y27632. This highlights a currently unmet demand for compounds that promote hPSC survival without inhibiting the ROCK signalling pathway.

1.4.1 Alternatives to ROCK inhibitors

A number of studies have been performed with the aim of discovering more specific or alternative pro-survival compounds. Despite screening over 20,000 molecules, a study performed by our group identified 18 molecules with any pro-survival effect on hPSC. It was subsequently shown by *in vitro* kinase assays that all of these compounds were inhibitors of ROCK (Andrews *et al*, 2010*)*.

In another high-throughput screen (50000 compounds), Xu and colleagues identified a further 2 compounds known as Thiazovivin (a 2,4-disubstituted thiazole) and Tyrintegin (a 2,4-disubstituted pyrimidine). The authors suggested that these compound supported increased survival by enhancing integrin signalling. However, upon further investigation, Thiazovivin was shown to act directly on the ROCK signalling pathway, significantly inhibiting ROCK activity at a concentration of 2μM. Although the *in vitro* kinase assay showed Tyrintegin was not an inhibitor of ROCK, the study did not further investigate this, which does not rule out the possibility of indirect inhibition. The supplementary data accompanying this article shows via TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) assay that inhibition of Pi3K signalling

ablated the positive effect of Tyrintegin which is consistent with the accepted role of Pi3K in apoptosis (Wong *et al*, 2007; Yamaguchi and Wang, 2001).

Alternative strategies to pharmacological prevention of apoptosis such as genetic manipulation of hPSC have also been investigated. It has been recently shown that the overexpression of BCL2 in either constitutively active or inducible systems was able to improve survival of hPSC post dissociation (Ardehali *et al*, 2010), however the practicality of such systems has to be questioned.

1.4.2 T16 discovery

During the aforementioned work performed by our group (Andrews *et* al 2010), although all 18 compounds were shown to be ROCK inhibitors, we identified an additional 19th compound that promoted hPSC survival, but *in* vitro kinase assays it was shown that this compound, referred to as T16, did not have any inhibitory effect on ROCK or the closely related kinase PRK2. Further characterisation of the compound was not within the scope of that project and T16 was therefore not included in the resulting publication.

1.5 Aims of thesis

The aim of this thesis was to further characterise the activity of the hPSC survival compound T16 and to demonstrate its potential as a novel reagent for use in hPSC culture and for the development of hPSC-derived cell therapies. This included:

- Optimisation of compound performance
    - Concentration and duration of treatment
    - Numerous cell lines including hiPSC
- Confirmation of retained stem cell identity
    - Differentiation capacity
    - Expression of pluripotency markers

    - Karyotypic stability

- Investigation of potential upstream or downstream interaction with ROCK signalling pathway

    - RhoA/Rac1 activity

    - MLC phosphorylation status

    - Morphology analysis

- Investigation novel, non-ROCK, mechanism of hPSC survival

    - mRNA analysis

    - Kinase inhibition assays

    - Pathway tracking

2 Materials and Methods

2.1 General laboratory practice

Laboratory reagents and equipment were of the highest commercially available standard. Unless otherwise stated, chemicals and reagents were supplied by Sigma-Aldrich, Dorset, UK. Hazardous chemicals were handled in compliance with Control of Substances Hazardous to Health (COSHH) guidelines. Laboratory coats, nitrile powder-free gloves, safety spectacles and fume hoods were used where appropriate.

2.2 Biological tissue handling

Tissue culture was performed in standard biological safety class II vertical laminar flow cabinets under sterile conditions. Cells were cultivated in humidified incubator at 37°C with a constant atmosphere of 5% CO_2. Cells were regularly tested for Mycoplasma using MycoSEQ Mycoplasma scan kit (Invitrogen) and independently tested for karyotypic abnormalities upon removal from liquid nitrogen and after each subsequent 15-20 passages (Medical Genetics department, Yorkhill Hospital, NHS Greater Glasgow & Clyde). Table 1.1 shows a list of commonly used tissue culture plastics and reagents.

Item	Supplier
1ml syringes	Fisher
Bijou	Fisher
cell lifters	Corning Incorporated
Cryovials	Starlab UK
Culture dishes (all sizes)	Corning Incorporated
Culture flasks (all sizes)	Corning Incorporated
DMSO	Sigma
Falcon tubes (15ml and 50ml)	Corning Incorporated
Foetal calf serum (FCS)	Life Technologies
Knockout DMEM	Life Technologies
L-glutamine	Life Technologies
low binding pipette tips	Scientific lab supplies
Mitomyocin C	Sigma
Non-essential amino acids (NEAA)	Life Technologies
PBS (with Mg2+ and Ca2+	Life Technologies
PBS (without Mg2+ and without Ca2+)	Life Technologies
Penicillin	Life Technologies
Pipette tips (all sizes)	Starlab UK
Reaction tubes (eppindorfs)	Starlab UK
Serological pipette (all sizes)	Corning Incorporated
Square petri dishes	Fisher
Stericup filter units (250ml and 500ml)	Millipore
Streptomycin	Life Technologies
Ultra low attachment dishes (6-well)	Corning Incorporated

Table 2.1- Commonly used tissue culture plastics and reagents.

2.2.1 Human pluripotent stem cell culture

2.2.1.1 Maintenance

HPSC were cultured in adherent conditions in a feeder-free system with media being replenished every 24hours (6 out of 7 days). Maintenance of hPSC was performed using StemPro hESC SFM culture medium (Life technologies) which consisted of DMEM F12 with GlutaMAX, 1x StemPro hESC supplement and 1.8% bovine serum albumin (BSA). This was supplemented with 0.1mM 2-mercaptoethanol and 20ng/ml bFGF. Cells were passaged mechanically using StemPro EZPassage disposable passage tools (Invitrogen) when approximately 80-

90% confluent at a split ratio of 1 in 6. EZPassage tools produce a grid like pattern of hPSC in small colonies. Tissue culture vessels were pre-coated with extracellular matrix proteins as detailed in table 1.2.

Throughout this study, a number of cell lines were used. The original Wisconsin hESC lines H1 and H9 (Thomson *et al*, 1998) and the GMP (good manufacturing practice) grade line RC9 (Roslin Cells LTD, Edinburgh) were used alongside the hiPSC line NMF-iPS6 (Sullivan *et al*, 2010).

ECM	Supplier	Volume used	Conc used	Incubation	Temperature
Cellstart	Life Technologies	750µl/well	N/A	2hr	37°C
Human Fibronectin	Merck Millipore	100µl/well	0.3mg/ml	20mins	37°C
Recombinant Vitronectin	Stemcell Technologies	500µl/well	25µg/ml	1hr	Room Temp
BD Matrigel	BD Biosciences	1ml/well	0.5mg/ml	1hr	Room Temp
Gelatin (Bovine skin)	Sigma	1ml/well	0.1% solution	1hr	Room Temp

Table 2.2- Commonly used extracellular matrices.

2.2.1.2 Single cell passage using survival compounds

For dissociation to single cell suspension from hPSC, culture medium was removed and the cells were washed with PBS (-/-) (Invitrogen). TrypLE Select (Invitrogen) was added to the cells and incubated for 3-5mins at 37°C before being inactivated by the addition of an equal volume of basal medium. Cells were vigorously pipetted to dissociate any remaining clumps, counted using the haemocytometer then pelleted by centrifugation for 3mins at 300 x g in a Heraeus Multifuge 3 S-R. Cells were washed in PBS (-/-) and pelleted as before. Cells were resuspended in culture medium and seeded onto pre-coated tissue culture plates at a density of $5x10^5$ cells/well of a 6 well culture dish.

As detailed in results chapter 3 the survival compounds T16 and Y27632 required different treatments in order to produce optimal results. Therefore, T16 and Y27632 were added to cells 24hr and 2hr prior to passage respectively.

For long term exposure experiments, cells were passaged once they reached 90-100% confluence which took between 3-5days.

2.2.1.3 Survival assay

Survival of hPSC upon dissociation to single cells was assayed 24hr post dissociation by counting cells that remained in suspension and subtracting these from the total cells input. This value was presented as a percentage of total cells input. This is a modified version of that used previously by Chen *et al* (2010).

$$\frac{\text{(Total cells input) - (cells in suspension)}}{\text{(Total cells input)}} \times \frac{100}{1}$$

1.5.1.1 Supply of T16

The structure of T16 was not disclosed during these studies, however the compound was identified from a screen of commercially available drug-like moieties and can be identified for future studies. The compound was synthesised when required by the chemical supplier Enamine (Ukraine) in quantities ranging from 10mg to 100mg and supplied as a lyophilised powder. T16 was reconstituted in DMSO at a stock concentration of 50mM and used routinely at 30μM.

2.2.1.4 Kinase inhibition studies

In order to investigate the role of particular pathways in dissociation induced apoptosis of hPSC, kinase inhibitors were utilised. Unless otherwise stated, kinase inhibitors were added to cells 2hrs prior to passage and also supplemented in culture medium post passage. A list of inhibitors used and concentrations is in table 1.3. Unless otherwise stated, DMSO was used to reconstitute all inhibitors and an equal amount of DMSO (always <0.01% v/v) was used as a carrier control.

2.2.1.5 RhoA and Rac1 inhibition and activation

To produce maximum effect, any compounds other than Y27632 that target RhoA or Rac1 signalling pathways were added to cells 24hours pre passage and culture medium also supplemented with compounds post passage. A list of inhibitors and activators used can be seen in table 1.3.

2.2.1.6 Haematopoietic differentiation of hPSC

Differentiation towards red blood cells (RBC) involved a 31day long multistage feeder-free protocol (Mountford lab, patent filed).

2.2.1.7 Passive multi-lineage differentiation

In order to confirm the pluripotent potential of hPSC, cells were passively differentiated as follows. One confluent well of cells had culture medium removed and cells washed twice in PBS (-/-). 2ml of differentiation medium consisting of Knockout DMEM, 20% FCS, 1% NEAA, 1% L-glutamine and 1% penicillin and streptomycin was added to cells. EBs were produced by mechanically passaging cells as described in section 2.2.1.1 and transferring 1ml of cells in to 2 wells of an ultra-low attachment culture dish. A further 2ml of differentiation medium was added to leave total volume of 3ml/well. Culture medium was partially replenished every 2-3days by removing 1.5ml and supplementing with 1.5ml of fresh differentiation medium, taking care to limit cell loss. After 2weeks in suspension culture, embryoid bodies were transferred to 12-well dishes that were pre-coated with 0.1% Gelatin for 1hour at room temperature using a split ratio of between 1in2 and 1in4. Cells were supplied with 3mls fresh differentiation medium every 2-3days for a further 2weeks before being fixed for subsequent staining as described in section 2.4.10.

Inhibitor/Activator	Target	Concentration used	Supplier
Lysophosphatidic Acid (LPA)	RhoA (Activator)	Various	Tocris bioscience
GF109203X	PKC (α & ß1 isoforms)	10μM	Tocris bioscience
API-2	AKT	10μM	Tocris bioscience
CGP53353	PKC (ß2 isoform)	10μM	Tocris bioscience
PI828	Pi3K	10μM	Tocris bioscience
Rho inhibitor 1	RhoA (inhibitor)	20ng/μl	Cytoskeleton Inc.
BIBX1382	EGFR	10μM	Tocris Bioscience
LY 364947	TGFß	10μM	Tocris Bioscience
PP2	Src-family kinases	10μM	Tocris Bioscience
Y27632 (in dH_2O)	ROCK	10μM	Sigma
Blebbistatin	Myosin II	10μM	Sigma
NSC23766	Rac1 (GEF inhibitor)	Various	Sigma
TPCA-1	IκK	10μM	Tocris Bioscience

Table 2.3- List of inhibitors and activators used. Unless otherwise stated, all inhibitors were made up in DMSO at stock concentrations which ensured that no more than 0.01% v/v of DMSO was added to cultures.

2.2.2 Tissue Culture

2.2.2.1 Mouse Embryonic Fibroblasts (MEFS) and media conditioning

Mouse embryonic fibroblasts or MEFS, were cultured in DMEM supplemented with 10% FCS, 5% P/S, 2mM L-Glutamate and 1mM sodium pyruvate. MEFS were grown until 80-100% confluent and either passaged or mitotically inactivated using

mitomyocin C. To inactivate MEFS, 10ng/ml mitomyocin C was added to culture medium and incubated with cells for 2-3hours. Culture vessels were pre-coated with 0.1% gelatin solution at room temperature for 1hour. Culture medium containing mitomyocin C was removed from the culture vessels and cells washed 3x in PBS (-/-) before being dissociated using TrypLE select. Cells were centrifuged at 300 x g for 3mins, resuspended in MEF media and replated onto the gelatin coated plates at a density of $6x10^4$ cells per cm^2. The following day, MEF media was removed, cells were washed 2x with PBS (-/-) and had the medium to be conditioned added (21mls per T150 culture vessel). At this point medium was supplemented with 4ng/ml of bFGF. Conditioned medium was harvested after 24hours and fresh medium added to MEFS. This was repeated 3-4 times as dictated by the quality of the MEFS. Prior to use, conditioned medium was filtered and aliquoted into working volumes. If not used immediately, conditioned medium was stored at -80°C. MEFS were not used beyond passage 5.

2.2.3 Cryo-preservation and recover of cells

Cells were treated as described in section 2.2.1.1 above. The small colonies created by the EZPassage cutting tool were resuspended in 1ml of Stempro medium and 500µl added to 500µl of a freezing mixture composed of 10% culture medium, 30% FCS and 10% dimethyl sulphoxide (DMSO). This 1ml cell and freezing mix solution was added to 1 cryovial and placed in a Nalgene® Mr. Frosty® container containing isopropanol and placed in the -80°C freezer. This ensured the gradual decrease of temperature towards -80°C at a rate of 1°C per minute. Vials were subsequently transferred to liquid nitrogen and stored until required.

Prior to removal from liquid nitrogen, pre-coated culture vessels were incubated at 37°C with culture medium for 30minutes. Cells were removed from liquid nitrogen and thawed quickly in a 37°C water bath until a single ice crystal remained. Cells were transferred to a 15ml tube and resuspended in the pre-incubated culture medium by adding a drop at a time. Cells were then transferred to culture dish and returned to incubator. Culture medium was replaced the following day to ensure removal of any residual DMSO.

2.3 Gene expression analysis

2.3.1 RNA extraction

Total RNA was extracted from cells through the use of the Qiagen RNeasy mini kit as per manufacturer instructions. Briefly, cells were lysed either directly on the plate or after being dissociated as described previously. In both cases, 350µl of Buffer RLT was used per confluent well of a 6 well dish. Cell lysis was aided through the use of a rubber policeman if directly lysed on the plate or via pipetting 3-5 times if already dissociated. Lysates were either directly processed or stored at -80°C until required. In order to optimise the concentration of RNA that binds to the spin column, lysates were homogenised by passing them through QIAshredder spin columns using a benchtop Heraeus BIOFUGE pico for 2mins at 10000 x g. RNA was precipitated by the addition of an equal volume of 70% ethanol. The sample was then passed through an RNeasy spin column containing a silica based gel membrane which binds the RNA for 15secs at 6000 x g. Membrane bound RNA was treated with 700µl Buffer RW1 by spinning it through the column for 15secs at 6000 x g. RNA was then washed twice in 500µl Buffer RPE by addition to the spin column followed by spinning for 15secs at 6000 x g and 2mins at 6000 x g for first and second washes respectively. RNA was then eluted by passing 30-50µl of RNase free water through the spin column for 1min at 6000 x g. The eluate can be frozen at -80°C at this stage or further processed.

2.3.2 DNase treatment

Digestion of contaminating DNA was performed using the Ambion Turbo DNA free kit. The reaction was initiated by adding 2µl buffer, 12µl water and 1µl DNase to 5µl RNA eluate and incubating this mixture for 30mins at 37°C. Samples were briefly centrifuged and another 1µl of DNase added for a further 30mins at 37°C. Samples had 2µl stop solution added and were left for 2mins at room temperature. Samples were centrifuged for 5mins at 6000 x g and the supernatant, which contains the DNased RNA was transferred to a fresh eppindorf and either used to produce cDNA or stored at -80°C for future use.

2.3.2.1 RNA Quality control

RNA concentration and quality was assayed through use of the NanoDrop (Thermo Scientific) and the Agilent® 2100 bioanalyser respectively. The Agilent produced RNA integrity numbers or RIN values that were used to determine quality. Values of 7 or more were deemed as being of high quality. Briefly, DNAse treated RNA was produced as above, with 1µl per sample being used for Agilent analysis. Agilent gel was prepared by centrifuging 550µl gel matrix through a spin column at 1500 x g for 10mins. 65µl of the eluate was transferred to a new eppindorf and had 1µl RNA 6000 Nano dye concentrate added and mixed well. This Gel-Dye mix was centrifuged for 10mins at 13000g. RNA 6000 nano chip was placed on the Agilent priming station and had 9µl Gel-Dye mix added and spread through the chip using a 1ml syringe attached to the priming station. After this another 9µl was added to two further wells. 5µl of RNA 6000 Nano marker was added to all 12 sample wells as well as the ladder well. 1µl sample was added to each of the 12 samples wells (or Nano marker if well not required) and 1µl ladder added to the ladder well. The loaded chip was placed in the vortexer for 1min at 2400RPM. The chip was then immediately run on the Agilent® 2100 bioanalyser. A single quantitative real time polymerase chain reaction, or qRT-PCR, reaction can also be performed using a stably expressed housekeeping gene with DNAse treated RNA to check for any contaminating DNA. This is performed as detailed in section 2.3.4.

2.3.3 cDNA synthesis

In order to analyse cellular gene expression, total RNA must first be used to create cDNA (complementary DNA). cDNA synthesis was performed by adding 10µl of DNase treated RNA to a reaction mix composed of 10mM dNTPs and 1µl of random hexamers (300ng/µl) (Invitrogen). The reaction was initiated by heating to 65°C for 5mins. Samples were chilled on ice, briefly centrifuged and combined with a reaction mixture containing 4µl buffer, 2µl DTT and 1µl RNaseOUT (Invitrogen) and left at room temperature for 2mins. 1µl of superscript II (Invitrogen) was added to samples before they were put through a temperature cycle consisting of 25°C for 10mins, 42°C for 50mins and 70°C for the remaining 15mins. As before, samples were then immediately used for analysis or frozen at -80°C.

2.3.4 TaqMan qRT-PCR transcript analysis

TaqMan based qRT-PCR can be used as an efficient and reproducible method of analysing gene expression. This technique utilises small sequence specific probes for genes of interest that have a reporter fluorophore at the 5' end and a quencher molecule at the 3' end. During the amplification process, if the target sequence is present, the probes anneal and the quencher is subsequently cleaved during the amplification process by the enzyme Taq polymerase which has exonuclease activity, functioning in the 5' to 3' direction. Cleavage of the quencher molecule allows for detection of the reporter fluorophore, with the signal intensity increasing after each successful amplification cycle being directly proportional to the starting concentration of target sequence. This fluorescence signal can be normalised to that of a housekeeping gene which should be a gene that retains steady expression independent of experimental procedures. This process allows for the presence of genes of interest to be detected and quantified. Unless otherwise stated, the housekeeper gene GAPDH was used throughout.

Samples were prepared for qRT-PCR analysis by adding 5µl of a 1 in 64 dilution (in water) of cDNA to a reaction mix containing 2.2µl H_20, 7.5µl 2x TaqMan Gene Expression master mix (ABI) and 0.3µl of primer. Reactions were performed in Optical 96-well Reaction Plates (ABI) with technical and biological replicates.

Data is analysed by comparisons between the cycle thresholds (C_t) for each probe measured to that of a housekeeper gene. GAPDH was routinely used given its stable expression with hPSC. C_t is measured when the reporter dye emission intensity rises above that of the background level which occurs during the exponential phase of PCR. These C_t values are used to generate a ΔC_t, which represents the difference between the gene C_t and the housekeeper C_t. The mean ΔC_t between replicates was calculated and was used to calculate the $2^{\Delta Ct}$ x 1000 value which was subsequently plotted on graphs. Using this this method, a relatively high $2^{\Delta Ct}$ x 1000 of 400, would be indicative of a signal being detected within 1 cycle of the housekeeper, whereas a value of less than 0.1 would result from a signal being detected more than 15 cycles after the housekeeper. This method is particularly useful to give an indication of the absolute level of expression and relative quantification when an appropriate denominator is not

available for the $2^{-\Delta\Delta CT}$ method, for example when a genes is not expressed in the control undifferentiated cells but is induced by treatment and therefore comparing expression would result in an infinite fold increase.

2.3.5 TaqMan Low Density Array cards (TLDA)

TaqMan low density array cards (TLDA) plates operate under the same principles as single assay qRT-PCR only on a higher throughput scale. This platform utilises micro fluidic plates capable of simultaneously performing 384 reactions without requiring large amounts of sample. The cards allow a maximum of 8 samples to be processed using only 9µl of cDNA per sample. Briefly, 9µl of undiluted cDNA was added to 45µl water and 54µl 2x TaqMan Gene Expression master mix. 108µl of this mixture was directly added to one port on the TLDA card and analysed using the 7900HT Fast Real-Time PCR System.

Data produced using TLDA was analysed as described above.

2.3.6 Microarray

Microarray analysis was performed in conjunction with the Glasgow Polyomics Facility using the Affymetrix HuGene ST 1.0 Human Array platform. RNA extraction was performed as discussed in section 1.3.1, however DNase treatment was performed on column through the addition of 10µl DNase and 70µl Buffer RDD directly onto the spin column membrane immediately following the Buffer RW1 wash step. This was incubated at room temperature for 15mins then washed again in Buffer RW1 before continuing with standard RNA extraction protocol starting with the first Buffer RPE wash step as described above. DNase treated RNA was processed on the Agilent and NanoDrop to ensure sufficient quality and concentration before continuing the protocol, with only samples obtaining a RIN value >7 being used.

Samples were further processed via the use of two separate kits. The first kit was the Ambion WT Expression Kit which produces sense strand cDNA by firstly adding 5µl RNA to 5µl of First-Strand Master Mix composed of 4µl First-Strand Buffer Mix and 1µl First-Strand Enzyme Mix and incubating for 1hr at 25°C followed by 1hr at 42°C and then 2mins at 4°C. Samples were briefly centrifuged

and allowed to cool on ice for 2mins before processing was continued by adding 50µl Second-Strand Master Mix which was composed of 32.5µl nuclease free water, 12.5µl Second-Strand Buffer Mix and 5µl Second-Strand Enzyme Mix. The First-Strand cDNA and Second-Strand Master Mix were put through a further temperature cycle this time consisting of 1hr at 16°C, 10mins at 65°C and 2mins at 4°C. Samples are once again centrifuged briefly before being cooled on ice. This Second-Strand cDNA was then used to produce anti-sense cRNA by incubating 60µl with 30µl of *In Vitro* Transcription (IVT) Mix composed of 24µl IVT Buffer Mix and 6µl IVT Enzyme Mix. This reaction mix was incubated for 16hrs at 40°C and then left overnight at 4°C. cRNA was then purified by adding 60µl cRNA Binding mix which was composed of 50µl Nucleic Acid Binding Buffer Concentrate and 10µl Nucleic Acid Binding Beads before transferring samples to a 96well U-Bottom Plate. Samples had 60µl of Isopropanol added and were subsequently placed on a plate shaker for 2mins, during which time the cRNA would bind to the beads. The plate was transferred to a magnetic plate which allowed the supernatant to be discarded. 100µl of Nucleic Acid Wash Solution was used to wash each sample 2x, again using the magnetic plate to capture beads. Washed cRNA was eluted from the beads by adding 40µl pre-warmed Elution Buffer (55°C) to samples for 2mins at room temperature followed by a further 3minutes on a plate shaker. The plate was returned to the magnetic plate to allow supernatants to be removed and transferred to fresh reaction tubes and placed on ice to cool. Sense strand cDNA is then synthesised by adding 10µg of cDNA to 2µl Random Primers and putting through a heat cycle of 5mins at 70°C, 5mins at 25°C and 2mins at 4°C. After completion of these incubations, 16µl of Second-Cycle Master Mix, which contains 8µl Second-Cycle Buffer Mix and 8µl Second-Cycle Enzyme Mix, was added followed by a further heat cycle of 10mins at 25°C, 90mins at 42°C, 10mins at 42°C, 10mins at 70°C and 2mins at 4°C. Samples were briefly centrifuged and kept on ice. Following this, 2µl RNase H was added to each sample and incubated for 45mins at 37°C, then 5mins at 95°C and 2mins at 4°C. This degrades the cRNA leaving the cDNA unaltered. cDNA was then purified using a cDNA Binding Mix and magnetic plate as described during cRNA purification step. Note that 120µl of ethanol is used rather than the isopropanol that was used during the cRNA purifying step. The eluted cDNA was transferred to fresh reaction tubes and stored on ice.

In order to fragment and label the cDNA, the Affymetrix GeneChip WT Terminal Labelling and Hybridization kit was used as per manufacturer instructions. Briefly, 5.5µg of single stranded, sense strand cDNA was incubated with 16.8µl of Fragmentation Master Mix ,which was composed of 10µl nuclease free water, 4.8µl 10x cDNA fragmentation buffer, 1µl of 10units/µl UDG and 1µl of 1000units/µl APE1, for 60mins at 37°C, 2mins at 93°C and 2mins at 4°C. 45µl of each sample was transferred to fresh reaction tubes, to which, 15µl of Labelling Reaction Mix (12µl 5x TdT Buffer, 2µl TdT and 1µl of 5mM DNA Labelling Reagent) was added and incubated for 60mins at 37°C, 10mins at 70°C and 2mins at 4°C. At this point samples were added to a Hybridization Mix (50pM Control Oligonucleotide B2, bioB, bioC and bioD Eukaryotic Hybridization Controls at concentration of 2.5, 5, 25 and 100pM respectively, 1x Hybridization Mix and DMSO) and incubated at 99°C for 5mins then 45°C for a further 5mins. Samples were centrifuged at full speed for 1minute before being loaded in to the HuGene ST 1.0 Human Array Chips. The loaded chips were transferred to a hybridization oven set at 45°C and spun at 60RPM for 17hrs. Chips were then processed using the Affymetrix GeneChip Scanner 3000 7G system.

2.4 Protein analysis

2.4.1 Protein extraction

As with RNA extraction, protein extraction was performed either directly on the plate or after dissociation as dictated by experimental design. Direct on the plate lysis was performed by removing culture medium and washing cells with ice cold PBS (-/-). 70-500µl lysis buffer # 6 (7.8ml H_2O, 2ml lysis buffer #6 concentrate (R&D), 3.604g urea, 100µl0.5M NaF) was added directly to wells and a rubber policeman was used to aid lysis. Lysates were then transferred to an eppindorf and gently agitated for 30mins at 4°C before being centrifuged at 8000 x g again at 4°C. These cleared lysates were transferred to a new reaction tube and snap frozen on dry ice and placed in -80°C freezer.

Cells that were harvested via dissociation were centrifuged at 8000 x g, washed in ice cold PBS (-/-) and centrifuged again with the same settings. Excess PBS was removed and cell pellet snap frozen on dry ice. Prior to use, cell pellets

were thawed and lysed by the addition of 70-500µl lysis buffer and treated as above.

For time course experiments, cells that had reattached to the ECM were lifted using a cell scraper before being treated as above. Unless kit specific protocols dictated otherwise, lysis buffer #6 (R&D), supplemented with protease and phosohatase inhibitors (Thermo Scientific), was used to lyse cells. All subsequent analysis steps were performed on ice and spins performed at 4°C.

2.4.2 Protein quantification (Bradfords Assay)

Protein concentration was determined by diluting samples 1 in 50 in water and 50µl added to triplicate wells of a 96 well culture dish (flat bottom). Protein standards ranging from 5µg/µl to 50µg/µl were also added in triplicate. To each well, 200µl Bradfords reagent (BIO-RAD) was added and samples immediately analysed via plate reader (BIO-RAD model680 microplate reader), reading absorbance at a wavelength of 595nm.

2.4.3 SDS PAGE

To ensure equal loading appropriate volumes of protein lysates (as calculated by Bradfords) were added to separate reaction tubes and made up to 24µl with water. Each sample had 6µl of 5x laemmli sample buffer added to give 1x final volume of 30µl. Laemmli buffer was composed of 10% SDS, 300mM Tris (pH 7.0), 0.05% Bromophenol blue, 50% glycerol and 10% ß-mercaptoethanol made up in dH_2O. Samples were boiled for 10mins with a brief vortex after 5mins. Samples were briefly centrifuged before being loaded into each well of a 4-12% Bis-Tris midi gel (Life Technologies). Novex pre-stained protein standards (Life Technologies) were loaded into one well. Gel electrophoresis was performed using the XCell SureLock Mini-Cell coupled with a power pack running at 200v for approximately 45mins. 1x MES or MOPS running buffer (Life Technologies) was used to aid gel electrophoresis.

2.4.4 Western blot

The proteins, now separated on the gel in a size dependent manner, were transferred to a nitrocellulose membrane (Sigma) with a 0.45µm pore size by western blotting. Transfer was performed for 90mins at 30v using an XCell II blot module and 1x transfer buffer (Life Technologies). Nitrocellulose membranes were then blocked for 30mins at room temperature using a 5% phosphoblocker solution (Cambridge Bioscience) made up in Tris buffered saline with tween (TBST). Primary antibody incubations were performed at 4°C overnight in a 1% phosphoblocker solution in TBST (Sigma-Aldrich). Table 2.4 shows a list of primary antibodies used. Membranes were washed three times with TBST for 5-10minutes and incubated for 90minutes at room temperature with suitable secondary antibody solution conjugated to horseradish peroxidase (HRP). Table 2.5 shows a list of secondary antibodies used. Membrane wash steps were repeated as described above before exposing the membrane to 6ml of Enhanced Chemiluminescence (ECL) reagent (Thermo Scientific) composed of equal parts of solutions A and B for 30seconds. Chemiluminescence was detected by developing films that had been exposed to the membrane using the X-OMAT. The resultant films were analysed using the densitometry analysis software Quantity One (BIO RAD). The protein α–tubulin was routinely used as a loading control due to its stable expression in hPSC. If required, nitrocellulose membranes were stripped by incubation at room temperature for 10-15mins with Restore Western Blot Stripping Buffer (Thermo Scientific) followed by a re-blocking step and subsequently probed for additional proteins.

Target Protein	Species Raised In	Dilution Used	Supplier
pMLC (ser19)	Rabbit	1 in 1000	Sigma
RACK1	Mouse	1 in 2000	SantaCruz
Src-family kinases (pan Src)	Rabbit	1 in 1000	Cell Signalling Technologies
pSrc-family kinases (Tyr416) (pan pSrc)	Rabbit	1 in 1000	Cell Signalling Technologies
RhoA	Mouse	1 in 500	Millipore
Rac1	Mouse	1 in 1000	Millipore
ILK	Mouse	1 in 1000	Cell Signalling Technologies
α-tubulin	Mouse	1 in 10000	Abcam
Fyn (Tyr416)	Rabbit	1 in 1000	Cell Signalling Technologies

Table 2.4- List of Primary antibodies used for western blot.

Fluorophore	Species Raised In	Dilution Used	Supplier
Anti-Rabbit IgG (whole molecule) peroxidase linked	Goat	1 in 10000	Sigma
Anti-mouse IgG (whole molecule) peroxidase linked	Goat	1 in 10000	Sigma

Table 2.5- List of secondary antibodies used for western blot.

2.4.5 RhoA and Rac1 Activation Assay

In order to determine levels of active GTP bound RhoA and Rac1 proteins, affinity binding based assays were used. Briefly, cells were stimulated as required (dissociated with and without drug treatments) and cells processed as described in section 2.4.1. 500µl MLB lysis buffer (kit specific, Millipore) supplemented with protease and phosphatase inhibitors was used to lyse cells in place of lysis buffer number 6. Lysates were incubated at 4°C for 30mins with gentle agitation then centrifuged at 8000 x g for 5mins. 50µl of lysate was taken

for analysis of total protein levels and the remaining 450µl used for the affinity binding assay (with a minimum protein concentration of 5mg/ml). Agarose beads bound to GST tagged fusion proteins that only bind active GTP bound RhoA or Rac1 were added to the lysate and incubated for 1hour at 4°C with gentle agitation. RhoA and Rac1 kits utilised rhotekin and PAK bound beads respectively. Samples were briefly centrifuged at 8000 x g and supernatant discarded. Beads containing only GTP bound RhoA or Rac1 were then washed in MLB buffer and centrifuged again at 8000 x g. This wash step was repeated 2 more times. After the final wash, extra care was taken to ensure removal of all the supernatant and the beads were then resuspended in 25µl of 2x sample buffer. 1µl of 1M DTT was added to samples to aid dissociation of RhoA or Rac1 from the beads and samples boiled for 10mins with a brief vortex after 5mins. SDS PAGE and Western Blots were performed as described in sections 2.4.3 and 2.4.4 respectively. Levels of GTP bound proteins were quantified using densitometry and normalised to total protein level (GTP and GDP bound protein). Total protein analysis was performed as described above (section 2.4.4).

2.4.6 G-LISA RhoA activation assay

The G-LISA RhoA activation assay is a luminescence based kit used to specifically assess levels of active GTP bound RhoA. Cells were stimulated as required and lysates produced as described in section 2.4.1. A kit specific lysis buffer was used, supplemented with protease and phosphatase inhibitors. Protein concentrations were determined by adding 20µl of lysate to 1ml of precision red protein assay reagent and absorbance measured at 600nm in a spectrophotometer. Absorbance values were multiplied by 50 to achieve protein concentrations in mg/ml. Samples were diluted to 0.5mg/ml using lysis buffer and mixed with equal volume of Binding Buffer solution. 50µl of this solution was immediately added to a well of the G-LISA assay plate and incubated for 30mins at 4°C on an orbital shaker at 400RPM. Wells were coated with a RhoA binding protein specific for GTP bound RhoA only. After 30mins unbound sample was removed and wells washed 2x in wash buffer. Antigen presenting buffer (200µl) was added to each well for 2mins before washing a further 3x with 200µl wash buffer per wash. Primary and secondary antibodies were incubated in the wells for 45mins at room temperature on an orbital shaker at 400RPM with 3x washes

performed between incubations. Wells were given 3 final washes after removal of the secondary antibody solution. Wells were then exposed to 50µl ECL reagent and immediately analysed via plate reader (POLAR star Omega). Results generated were in RFU (Relative fluorescence units) and expressed as a percentage of the positive control. Positive control was performed as above, using recombinant RhoA protein supplied within the kit rather than protein sample.

2.4.7 Co-immunoprecipitation of RACK1 and Src

In order to assess intracellular protein to protein interactions, co-immunoprecipitations were performed. Cells were stimulated/treated as required and cell pellets generated and snap frozen as described in section 1.4.2. During cell lysis, it is critical that protein to protein complexes are not disrupted. In order to do this, a non-reducing lysis buffer was used in combination with sonication. Briefly, 500µl of non-reducing lysis buffer (Cell Signalling Technology) supplemented with protease and phosphatase inhibitors was added to cell pellet and titrated vigorously. Lysates were then sonicated on ice for 10 seconds and subsequently centrifuged at 6000 x g for 5mins. Supernatant was removed and transferred to a fresh tube on ice. At this point, a small aliquot was taken for analysis of total protein levels via SDS PAGE and western blot as described above. The remaining samples had 10µl of RACK1 primary antibody added and were incubated with gentle agitation for 1-2hours at 4°C. Following this, samples had 20µl of protein G agarose beads added using a pipette tip with 5mm cut off the top to avoid the shearing of beads. Samples were incubated for a further hour at 4°C with gentle agitation. During this time, protein G agarose beads, which are coated with covalently bound recombinant protein G with high affinity for IgG subclasses, binds to the IgG portion of the primary antibody. The RACK1 antibody in turn binds the specific epitope of RACK1 which will still be linked to any binding partners. Protein G agarose beads were pelleted by brief centrifugation at 6000 x g and supernatant discarded. Beads were washed by resupending in 500µl non-reducing lysis buffer. This step was repeated a further 2 times with extra care being taken after the third wash to remove as much supernatant as possible. 25µl of sample buffer (as described in section 2.4.3) was added to each sample before being boiled for 10mins with

a brief vortex after 5mins. SDS page and western blot were performed and samples analysed by densitometry as described previously.

2.4.8 GST RACK1 fusion protein

2.4.8.1 Expression and purification

GST RACK1 fusion protein was generated from frozen glycerol stocks of *e.coli* containing pGEX-RACK1. Briefly, 50ml of L-broth was inoculated with pGEX-RACK1 *e.coli* and incubated overnight in a shaker maintained at 37°C. The following day, the overnight culture was used to inoculate 450ml of L-broth containing 100µg/ml ampicillin. Cultures were grown at 37°C in the shaker until they had an OD_{600} of between 0.6-1.0 as determined by spectrophotometer readings taken after each hour of incubation. Once within this range, 0.2mM of IPTG (isopropylthio-ß-galactoside) was added to induce production and cultures incubated for another 4hours at 30°C. Cultures were then pelleted by centrifugation at 5000x g for 10mins at 4°C and stored at-80°C overnight. Cells were then thawed and resuspended in 10ml of ice cold resuspension buffer (50mM TrisCl pH 8, 10mM NaCl, 1mM EGTA, 1.92mM $MgCl_2$, 1mM DTT) supplemented with protease and phosphatase inhibitors. This solution was then sonicated on ice 4 times for 30seconds each, with a 30second gaps between each cycle, then had a 1 in 500 dilution of 10% Triton X-100 added. This mixture was vortexed then centrifuged for 15mins at 6000 x g at 4°C. Supernatant was removed and transferred to a 15ml tube and had 1ml of glutathione beads that were pre-equilibrated in wash buffer comprised of resuspension buffer and 0.02% Triton X-100. This solution was incubated for 1hr at 4°C with gentle agitation. Glutathione beads, now with GST-RACK1 bound to them were centrifuged at 4°C for 2mins at 600 x g and supernatant discarded. Beads were then washed in 5ml of wash buffer, centrifuged again as described above and then transferred to a fresh 2ml tube on ice. A further 3 washes were performed using 1ml wash buffer and centrifuged as previous. The incubation of beads with 600µl elution buffer (10mM glutathione in 50mM Tris pH 8) for 20mins at 4°C with gentle agitation facilitated the release of GST-RACK1 from the glutathione beads. Beads were then pelleted by a 10second full speed centrifugation and supernatant containing GST-RACK1 transferred to fresh tube on ice. This step was repeated two more times and eluates pooled before being transferred to a dialysis

cassette. Dialysis cassettes containing the eluted GST-RACK1 were added to 650ml of chilled dialysis buffer (50mM TrisCl Ph 8, 100mM NaCl and 5% glycerol) and incubated over night with three buffer changes. This allowed glutathione to diffuse into the buffer whilst keeping the GST-RACK1 within the dialysis cassette. This was then removed and GST-RACK1 stored at -80°C for future use. Concentration of GST-RACK1 was quantified via Bradford's assay as described in section 2.4.2.

2.4.8.2 Src pulldown using GST-RACK1 fusion protein

GST-RACK1 fusion protein was used as a tool to investigate RACK1 interaction with Src in response to various drug treatments. In order to do this, cells were treated as described previously and cell pellets generated. Cells were lysed with non-denaturing lysis buffer as described in section 2.4.7. As Src is unable to bind unphosphorylated RACK1, an in vitro phosphorylation reaction was performed by adding 30µl of GST-RACK1 and 40µl 1x mix of kinase buffer and ATP (Cell Signalling Technology) to 400µl of lysate. This mixture was incubated at 37°C with gentle agitation for 45mins then samples were stored on ice. Glutathione beads (30µl) were added to each sample before being incubated at 4°C for 1 hour with gentle agitation. The glutathione beads, now bound to GST-RACK1, were pelleted by brief centrifugation at 600 x g and washed three times in non-reducing lysis buffer (Cell Signalling Technology) with the supernatant being discarded each time. Beads were then resuspended in 25µl 2x sample buffer and boiled for 10minutes with a brief vortex after 5mins. Samples were processed by SDS PAGE and Western Blot as described previously.

2.4.9 Proteome Profiler™ antibody kits

Human Phospho-MAPK and Human Phospho-Kinase Proteome Profiler antibody arrays (R&D) utilise immobilised capture antibodies spotted in duplicate onto nitrocellulose membranes in combination with a cocktail of biotinylated detection antibodies. Signals are detected through the use of streptavidin-HRP conjugate and ECL reagent. Arrays were performed as per manufacturer's instructions. Briefly nitrocellulose membranes were incubated for 1hr at room temperature on a rocking platform with Array Buffer 5 which acts as a blocking buffer. 500µg of protein lysates prepared as described previously and made up to

a volume of 1.5ml in Array Buffer 1 were incubated with 20µl Detection Antibody Cocktail for 1hour at room temperature. After the hour incubation, Array Buffer 5 was removed from the nitrocellulose membranes and replaced with lysate-antibody mixture and incubated overnight at 4°C on a rocking platform. Membranes were washed 3 times in 1x Wash Buffer for 10mins per wash. Membranes were then incubated with 2ml of Buffer Array 5 and streptavidin-HRP mixture for 30mins at room temperature followed by a further 3 washes. Membranes were exposed to ECL reagent for 1minute and detected as described previously. Densitometry was used to measure signal intensity.

2.4.10 Immunocytochemistry

Cells grown on either culture plates or slides had culture medium removed, were washed 3x in PBS (-/-) and then had 4% paraformaldehyde (PFA) added (200µl/well of 12 well dish) for 20mins at room temperature. PFA was removed and cells washed a further 3x in PBS (-/-). After the final wash, a small volume of PBS was used to cover cells. Culture plates were sealed with parafilm and could be stored at 4°C before staining was performed. To continue with the staining protocol, PBS was removed and 200µl of permeabilisation solution (10% goat or donkey serum, 1% BSA, and 0.01% Triton X-100 made up in PBS (-/-)) was added to the cells for 30mins on a rocking platform. Following this, permeabilisation solution was removed and primary antibody added for 1-2hr at room temperature. Primary antibodies were diluted in permeabilisation solution and 200µl used per well. Cells were then washed 3x in PBS (-/-) for 5mins each on a rocking platform. Fluorescently conjugated secondary antibodies were added, again diluted in permeabilisation solution and incubated for 1 hour at room temperature on a rocking platform. During the secondary antibody incubation and all subsequent steps, culture vessels were wrapped in foil to avoid photo bleaching. The wash step was repeated, taking extra care to ensure all PBS was removed after the final wash. At this point 1 drop of Prolong Gold with DAPI (Invitrogen) was added to the cells and an appropriately sized glass cover slip placed on top with care being taken to avoid bubble formation. Images were produced using the Zeiss Axiovert 200M microscope and camera. Tables 2.6 and 2.7 show lists of primary and secondary antibodies used for immunocytochemistry.

Target	Species Raised In	Dilution Used	Supplier
E-Cadherin	Rabbit	1 in 200	R&D systems
Oct 4	Rabbit	1 in 200	R&D systems
Nanog	Rabbit	1 in 1000	Cell Signalling Technology
PAX6	Mouse	1 in 500	Sigma
AFP	Mouse	1 in 500	Abcam
SMA	Rabbit	1 in 200	Sigma
F-actin (FITC conjugated phalloidin)	N/A	1 in 200	Invitrogen
Tra-1-81	Mouse	1 in 200	Abcam

Table 2.6- Primary antibodies used for immunocytochemistry.

Fluorophore	Species Raised In	Dilution Used	Company
Alexafluor-555	Goat Anti-Mouse	1 in 400	Invitrogen
Alexafluor-488	Donkey Anti-Goat	1 in 400	Invitrogen
Alexafluor-488	Goat Anti-Rabbit	1 in 400	Invitrogen
Alexafluor-555	Goat Ant-Rabbit	1 in 400	Invitrogen

Table 2.7- Secondary antibodies used for immunocytochemistry.

2.5 Flow Cytometry

Cells were prepared for analysis by dissociating to single cell as described previously. Single cells were washed 3x in PBS (-/-), counted and resuspended in FACS sheath fluid (BD Biosciences). $1x10^5$ cells were transferred to FACS tubes and stained with required antibody by addition of 1µl antibody per $1x10^5$ cells. Antibodies were incubated for 30mins at room temperature and were protected from light. Cells were washed 3x in 2ml of sheath fluid 2mins of 300 x g centrifugation used to pellet cells between washes. Cells were finally resuspended in 500µl sheath fluid and analysed using the FACS Canto II with a

two laser setup (Red and Blue lasers). Subsequent analysis was performed using FloJo analysis software (Tree Star). Table 2.8 shows a list of antibodies used.

2.5.1 Apoptosis assays

Flow cytometry can be used as a means of determining if cells are healthy, necrotic, early apoptotic or late apoptotic by staining for the cell membrane phospholipid phosphatidylserine (PS) in combination with the nucleic acid dye propidium iodide (PI). One of the early steps of apoptosis is the translocation of PS from the intracellular to extracellular side of the plasma membrane. Annexin V has high affinity for PS and can be conjugated to fluorochromes such as FITC (Fluorescein isothiocyanate) that can be subsequently detected via flow cytometry. Those cells staining double positive for Annexin V and PI are late apoptotic as they have also lost membrane integrity. Cells staining positive for PI only are deemed to be necrotic as they have lost membrane integrity without actively apoptosing. Cells staining positive for Annexin V only are early apoptotic as they still retain membrane integrity. The BD Biosciences FITC Annexin V Apoptosis Detection Kit II was used as per manufacturer instructions. Briefly, cells were washed 2x in PBS (-/-) before being dissociated to single cell as discussed previously. After a further 2 washes, cells were resuspended in 1x Binding Buffer at a cell density of $1x10^6$/ml. 100µl of cell Binding Buffer solution was added to each reaction tube and incubated with 5µl of both FITC Annexin V and PI for 20mins at room temperature and protected from light. Samples were made up to 500µl by the addition of 400µl Binding Buffer and analysed using the FACS Canto II.

Target Protein	Species Raised In	Conjugate	Supplier
SSEA4	mouse	APC	BD Biosciences
Tra-1-60	mouse	PE	BD Biosciences
Tra-1-81	mouse	PE	BD Biosciences
SSEA1	mouse	FITC	BD Biosciences
SSEA3	mouse	PE	eBioscience
Annexin V	mouse	FITC	BD Biosciences
Integrin α2	mouse	APC	eBioscience
Integrin α4	mouse	PE	BD Biosciences
Integrin α5	mouse	APC	eBioscience
Integrin αv	mouse	PE	eBioscience
Integrin α6	mouse	PE	eBioscience
Integrin β1	mouse	FITC	BD Biosciences
Integrin β2	mouse	FITC	BD Biosciences
Integrin β3	mouse	PE	eBioscience
Integrin β5	mouse	PE	eBioscience
CD34	mouse	APC	eBioscience
CD36	mouse	PE	BD Biosciences
CD45	mouse	APC	BD Biosciences
CD71	mouse	APC	BD Biosciences
GlyA	mouse	PE	eBioscience
CD31	mouse	PE	BD Biosciences
CD43	mouse	APC	eBioscience

Table 2.8- Antibodies used during flow cytometric analysis.

1.6 Statistical analyses

Where appropriate, data was presented as mean values ± standard error of the mean (SEM). Experimental analysis between multiple treatment groups was performed by repeated measures analysis of variance (ANOVA). To determine statistical significance, Tukey post-hoc testing was performed, with the cut off for significance being $P=<0.05$. Statistical analysis was performed using GraphPad Prism software.

3 Characterisation of T16 as a Survival Molecule

3.1 General Introduction

T16 was originally identified using a high throughput screen that used a set concentration of 30μM in combination with a feeder-free culture medium composed of an equal volume of CM and a chemically defined medium with FN used as an ECM. In order to further investigate and optimise the performance of T16, these parameters were expanded and the usefulness of T16 as a survival compound tested.

The aims of these studies were to:

- Investigate the capacity of T16 to promote survival in multiple culture mediums, including more chemically defined options.
- Investigate T16 compatibility with multiple ECM components.
- Directly compare the survival mediated by T16 to that of Y27632.
- Investigate the capacity for long term and continuous use of T16.
- Determine if T16 use has any detrimental effect on the stem cell identity (retention of pluripotency markers, differentiation capacity and karyotypic stability).

3.2 T16 Concentration and Pre-Treatment Length

A variety of hPSC lines including the hESC lines H1, H9 and RC9 as well as an hiPSC line (NMF-iPS6); Sullivan *et al*, 2010) were tested over various concentrations including 10μM, 20μM, 20μM, 40μM and 50μM, as well as different pre-treatment durations ranging from 2hrs to 24hrs. Cells were enzymatically passaged as described in section 2.2.1.2, and assayed for survival 24hrs post passage. The data presented in figure 3.1 shows that T16 improves cell survival when compared to untreated cells in all but the 50μM samples. There is a significant increase in cell survival versus the untreated control cells when cells are treated with 10, 20, 30 or 40μM of T16 (P=<0.001). However, a treatment of 30μM resulted in significantly higher cell survival than any other concentrations (P=<0.001). There is no significant difference between untreated cells and cells treated with 50μM T16 which suggests that at this high concentration, T16 may be toxic to cells. T16 produced a similar pro-survival effect between all hPSC cell lines tested with there being no significant difference between these cell lines.

The data shown in figure 3.2 demonstrate that T16 treatment produced significantly increased cell survival when added to the cells 24hr prior to cell passage (P=<0.005). The general trend was towards increasing cell survival the longer the hPSC were exposed to T16, with all pre-treatment lengths producing significantly greater hPSC survival than cells with no pre-treatment (P=<0.001). Again there were no differences between cell lines used.

Based on the data shown in figures 3.1 and 3.2, all subsequent experiments were performed using a concentration of 30μM and with a 24hr pre-treatment.

3.2.1 Compatibility with Multiple ECM and Culture Mediums

There are a number of culture mediums and ECM available, each of which has its own pros and cons. To determine whether the pro-survival effect of T16 treatment was reproducible regardless of the ECM or culture medium used further experiments were performed. HPSC were treated with T16 then enzymatically passaged on to either Matrigel, fibronectin, CellStart or vitronectin (human recombinant) at a cell density of $5x10^5$ cells per well of a 6-

well culture dish with Stempro SFM used as the culture medium. Similarly, hPSC were passaged in to several different culture mediums including Stempro SFM, mTeSR and MEF CM, under standard conditions (fibronectin used as ECM with each media type). HPSC survival was assayed 24hrs post passage into the various culture mediums and ECM conditions. T16 produces similar cell survival regardless of the ECM or culture medium used (figure 3.3). Furthermore, there were no significant differences between cell lines used.

Unless otherwise stated, all subsequent experiments were performed using Stempro SFM in combination with fibronectin.

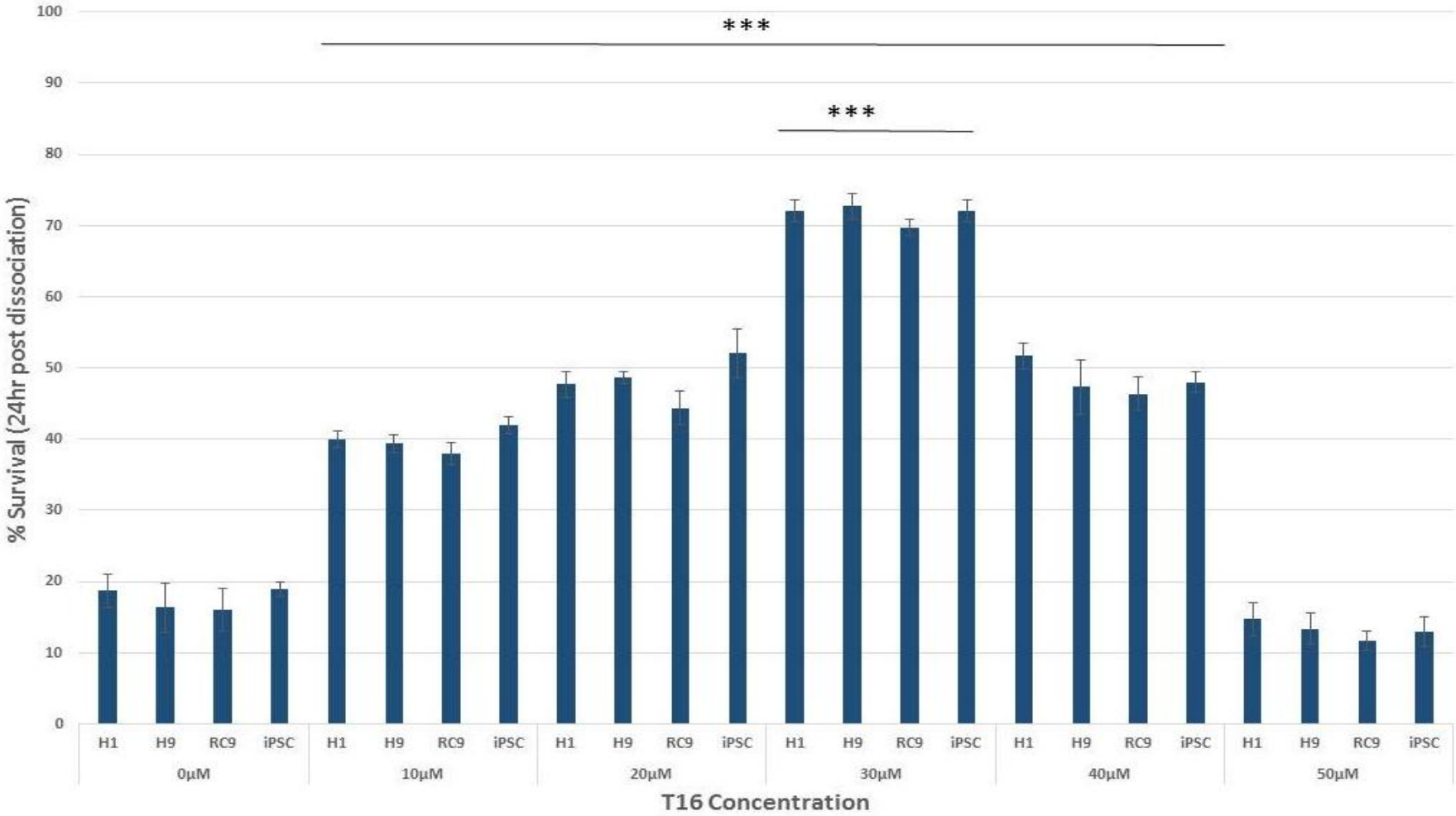


Figure 3.1- Optimisation of T16 concentration. HPSC were treated with varying concentrations of T16. Using a concentration of 10, 20, 30 or 40μM T16 resulted in significantly increased cell survival compared to either 50μM or untreated cells (P=<0.001). Cell survival after a 30μM treatment produced cell survival significantly higher than all other concentrations (P=<0.001). There was no significant difference between cell types. Data shown is the mean survival ±SEM, n=3.

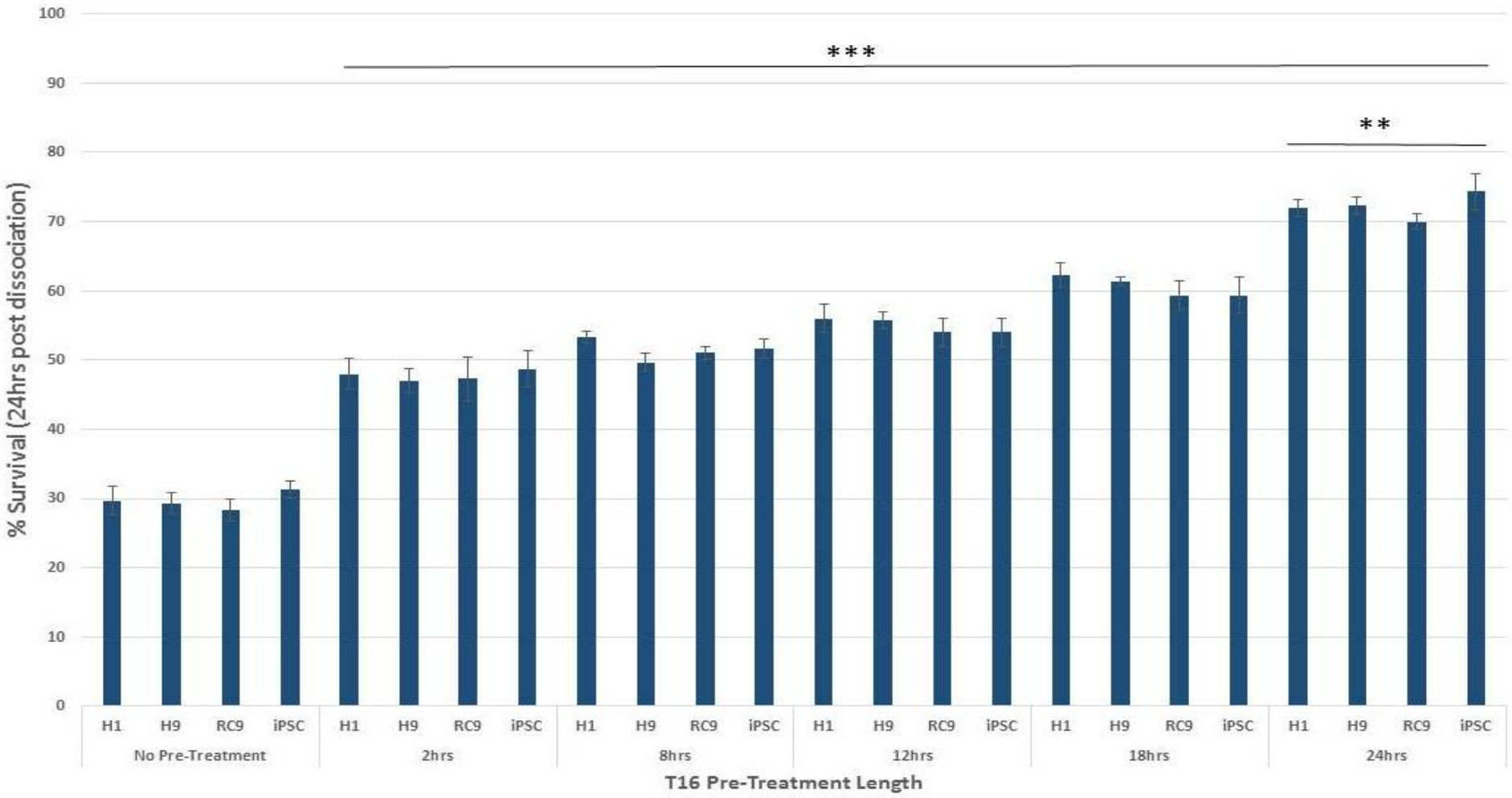


Figure 3.2- Optimisation of T16 pre-treatment length. HPSC were pre-treated with T16 for various time lengths prior to dissociation and then assayed for survival 24hrs later. Increasing length of time corresponded with an increase in cell survival, with all pre-treatment lengths producing significantly increased survival versus no pre-treatment (P=<0.001). The optimum pre-treatment was 24hr, which produced significantly increased hPSC survival compared to all other treatments (P=<0.005). There were no significant differences between cell lines. Data shown is the mean survival ±SEM, n=3.

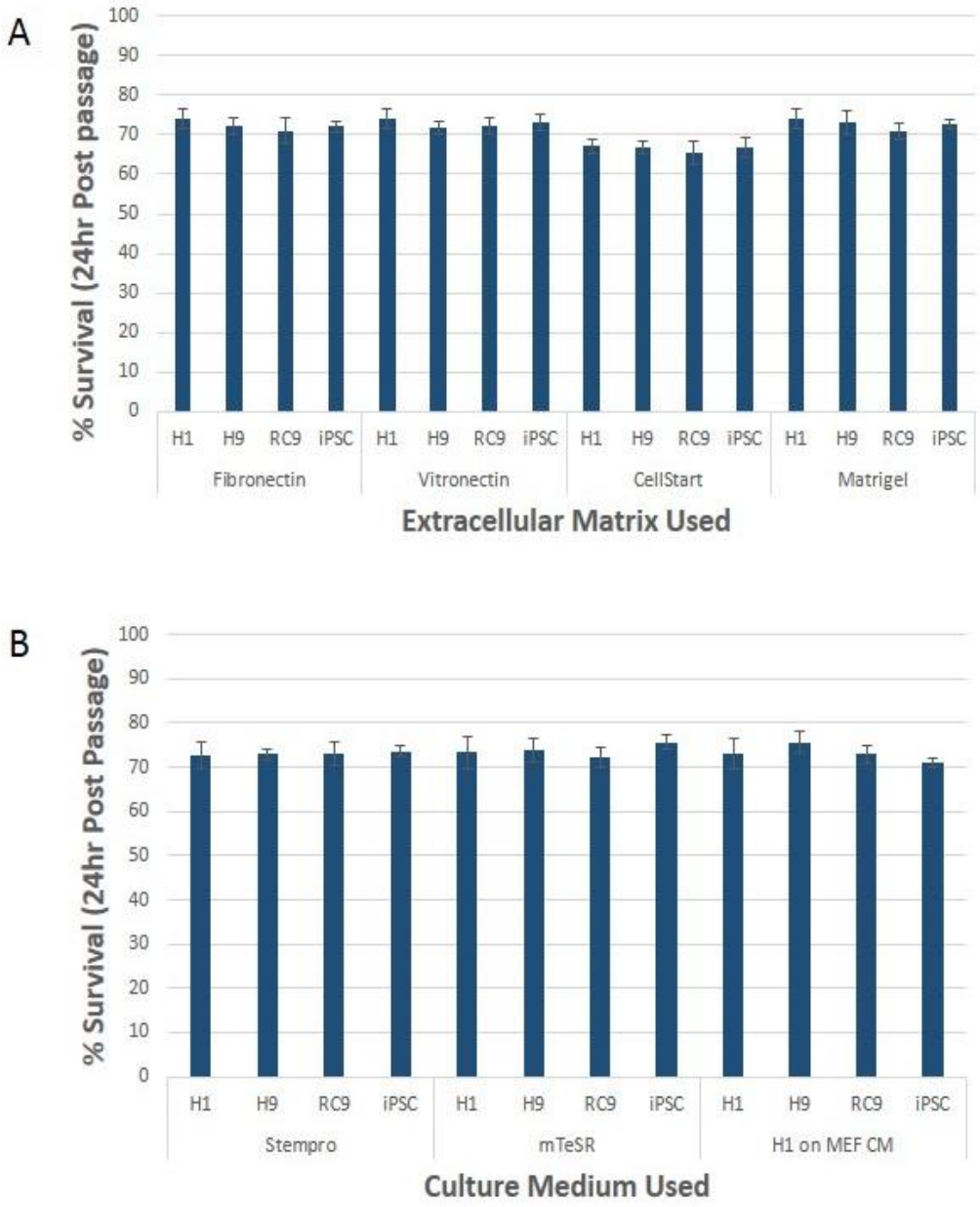


Figure 3.3- Compatibility with multiple extracellular matrices and culture mediums. HPSC were pre-treated with T16 and then passaged onto either fibronectin, vitronectin, CellStart or Matrigel using Stempro as the culture medium (A) or on to fibronectin with either Stempro SFM, mTeSR or MEF CM used as the culture medium (B). No condition produced cell survival that was significantly different. Data shown as mean survival ± SEM, n=3.

3.2.2 T16 treatment results in cell survival comparable to Y27632

The survival compound Y27632 has been used successfully as a survival compound since its initial description in 2007 (Watanabe *et al,* 2007) so for any alternative to be seen as acceptable, it must be shown to support survival just as effectively. HPSC were either treated with T16, Y27632 or left untreated, before being enzymatically passaged. In all experiments untreated cells had an equivalent volume of DMSO added as a carrier control, with cell survival being assayed 24hrs post passage. HPSC treated with either T16 or Y27632 had average cell survival that was significantly higher than that observed in untreated controls (P=<0.001) (figure 3.4) regardless of cell line used. In H1 hESC T16 treatment resulted in cell survival of 72% (±2.9), Y27632 produced survival of 72% (±3.6) and untreated cells had only 18% survival (±2.2). In the hESC lines H9 and RC9 and iPSC line NMF-iPS6, T16 treatment resulted in cell survival of 73% (±2.8), 69% (±1.9) and 74% (±2.6) respectively. In the same cell lines Y27632 produced cell survival of 73% (±2.1), 71% (±1.2) and 74% (±4.0) and DMSO alone (untreated) cells had survival of 18% (±2.4), 15% (±3.0) and 19% (±2.4) respectively. This result was consistent regardless of cell line used. These results show that a 24 hour pre-treatment with30μM T16 supports the enzymatic passage of hPSC as effectively as the well-established survival compound Y27632.

3.3 T16 can be used long term as a survival compound

In order for T16 to be considered as a viable alternative to Y27632, it must be shown that cells treated with T16 are able to support long term growth of hPSC in a manner comparable to, or better than, Y27632 treated cells. The hESC cell line H1 was enzymatically passaged 30 consecutive times (over approximately 20 weeks) using either T16 or Y27632 pre-treatment to support survival. Cells were passaged at 100% confluence which was approximately every 4-5 days and re-plated at a density of $5x10^5$ cells per well. HPSC survival was assayed 24hrs post passage each time. Data for untreated cells was only available after the initial passage due to the previously documented poor survival when no compound was added. T16 treated cells show similar survival to those treated with the commonly used Y27632, with there being no significant difference between

treatment types at any of the passages (figure 3.5). Similarly, no significant differences were observed when the experiment was repeated with a hiPSC cell line (Figure 3.6).

T16 and Y27632 treated cells reached confluence on the same day throughout these experiments. As the seeding density was constant and the survival was not significantly different, this suggest similar growth rate between treatment types.

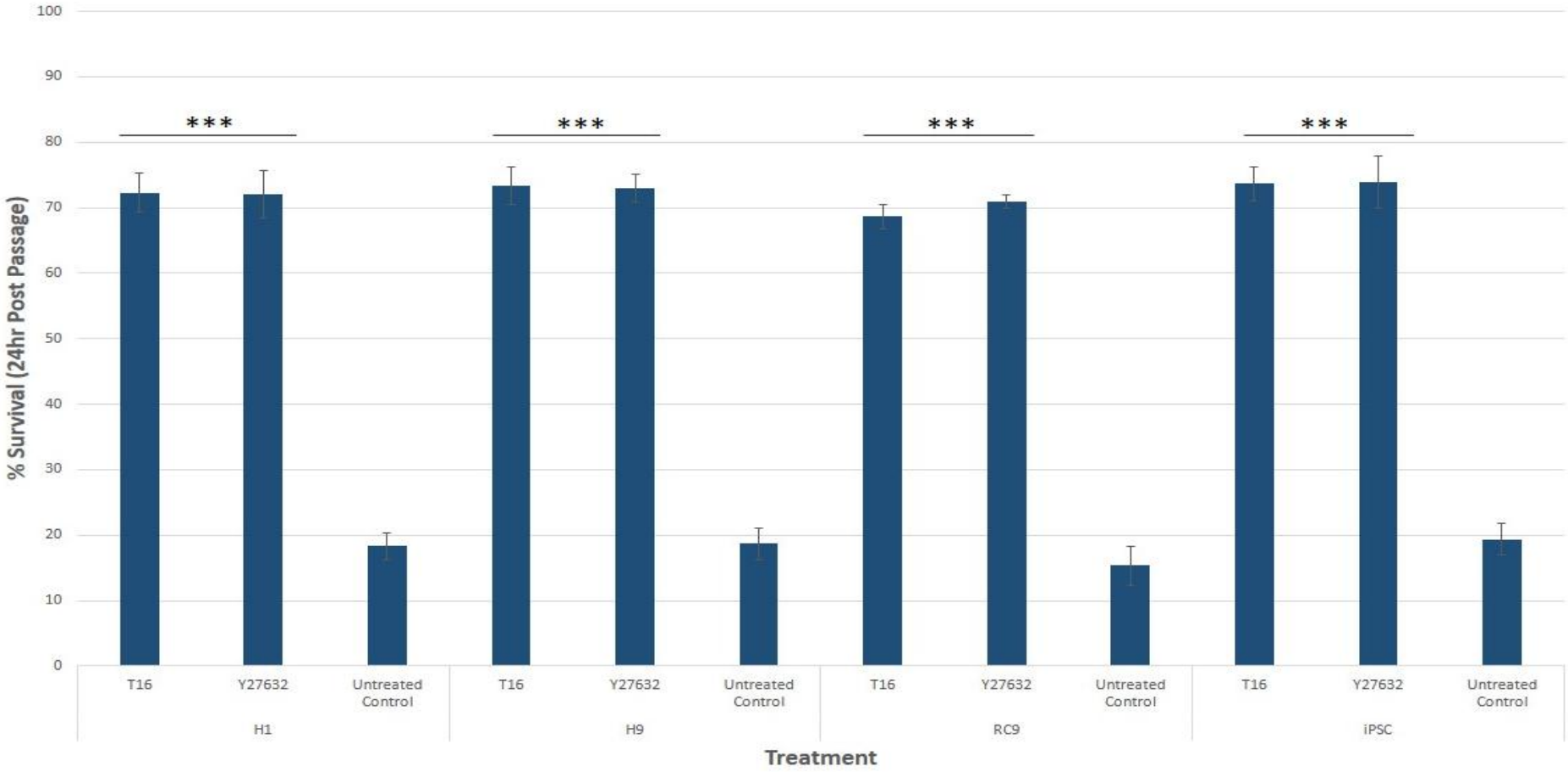


Figure 3.4-T16 supports survival as well as Y27632. HPSC were enzymatically passaged without a survival compound, or after treatment with either T16 or Y27632. The hESC lines H1, H9 and RC9 and the hiPSC NMF-iPSC6 had cell survival of 72% (±2.9), 73% (±2.8), 69% (±1.9) and 74% (±2.6) respectively when treated with T16. In the same lines, Y27632 resulted in cell survival of 72% (±3.6), 73% (±2.1), 71% (±1.2) and 74% (±4.0) and DMSO alone resulted in survival of 18% (±2.2), 18% (±2.4), 15% (±3.0) and 19% (±2.4) respectively. The survival produced in response to treatment with T16 or Y27632 was significantly higher than those of untreated cells (P=<0.001). Data shown is the mean survival ±SEM, n=3.

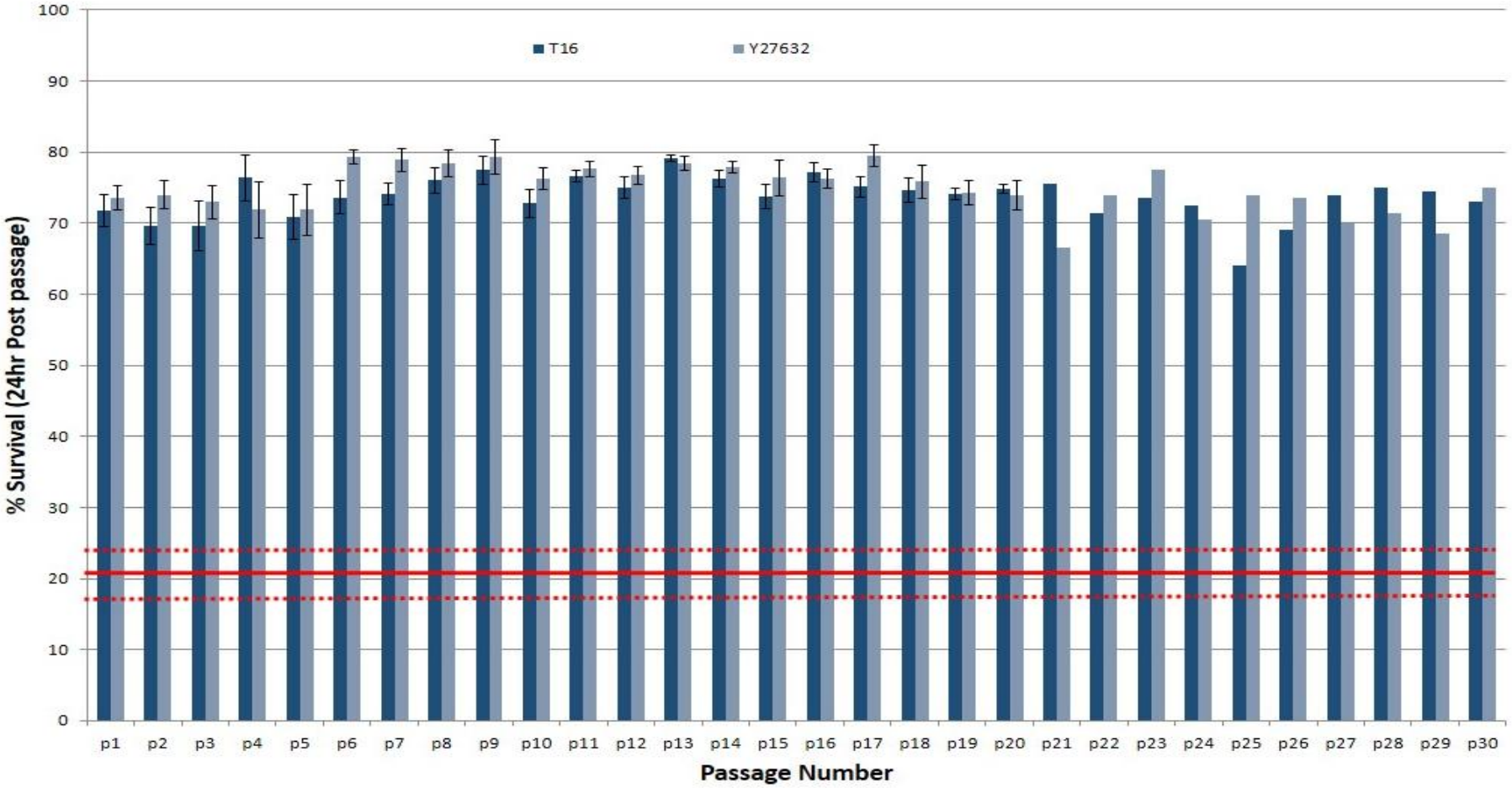


Figure 3.5- HPSC survival after long term exposure to survival compounds. T16 or Y27632 was used to enzymatically passage the hESC line H1 for 30 consecutive passages. Survival was assayed 24hrs post passage. Data up to passage 20 represents mean survival ± SEM, n=3. Data after passage 20 represents cell survival, n=1. The red line represents the average survival of untreated cells ± SEM, n=3

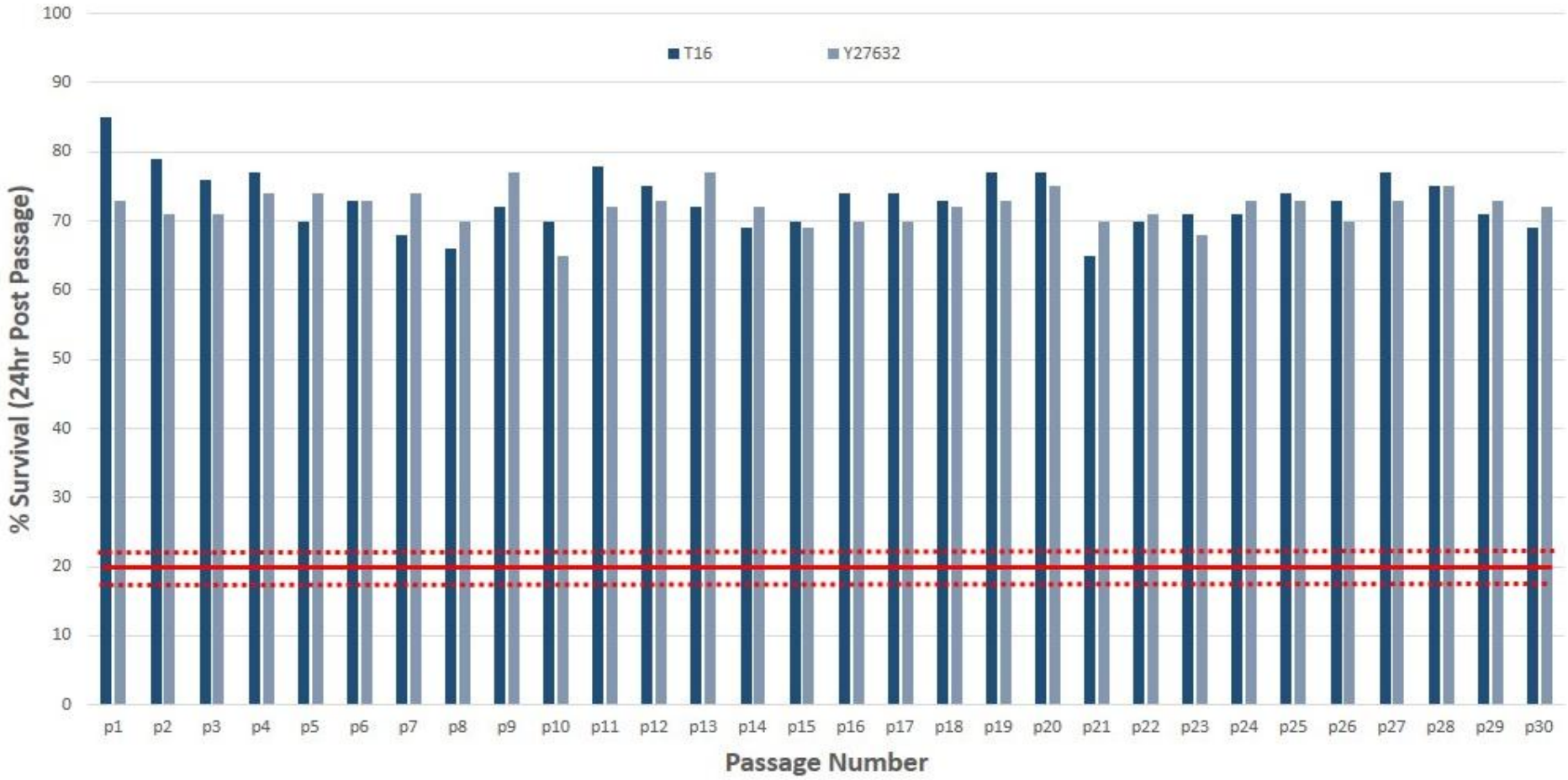


Figure 3.6-Long term survival produced using hiPSC. Cells were treated with either T16 or Y27632 and passaged enzymatically for 30 consecutive passages. Data represents survival from a single experiment (n=1). The red line represents the average survival of untreated cells ± SEM, n=3.

3.3.1 T16 treated cells retain cytogenetic stability

In order to ensure that T16 treatment was not causing karyotypic abnormalities or positively selecting abnormal cells during enzymatic passage, karyotypic stability was routinely tested by G-band analysis. Cells that had been enzymatically passaged with survival factors for the required number of consecutive passages were delivered to Medical Genetics department at Yorkhill Hospital (NHS Greater Glasgow & Clyde) at approximately 50-70% confluence. Cells were karyotyped after 12, 20 and 30 consecutive passages. Both T16 and Y27632 treated cells retained normal karyotype of 46 XY for at least 30 consecutive passages (Figure 3.7).

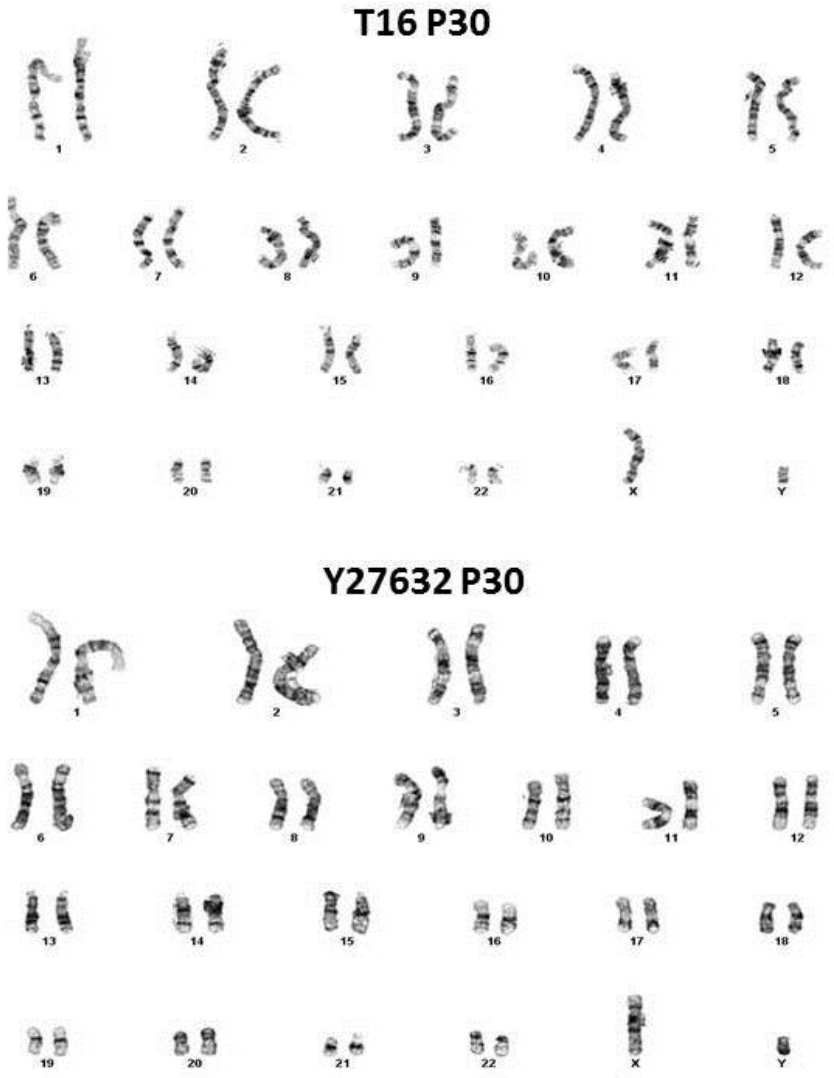


Figure 3.7-Cytogentic stability of T16 treated cells. HPSC (H1 hESC) treated with T16 or Y27632 for 30 consecutive passages were independently tested for cytogenetic stability (Yorkhill hospital, NHS Greater Glasgow and Clyde). Cells remain karyotypically normal (46, XY) until at least P30.

3.3.2 T16 treatment does not alter expression of pluripotency markers

A critical requirement of any reagent to be used for hPSC maintenance and culture is that it does not negatively impact the expression of pluripotency related genes. The long term maintenance of a range of pluripotent markers was determined by flow cytometry, immunocytochemistry and by mRNA transcript analysis.

3.3.2.1 Flow cytometric analysis of pluripotency markers

HPSC were analysed by flow cytometry after every 5 passage as described in section 2.5. Cells were harvested for analysis immediately following passage. Figure 3.8A shows representative data produced using the FACS Canto, with the left hand panel showing forward scatter on the X-axis and side scatter on the Y-axis, the centre panel showing an isotype control sample, gated to include >97% of cells in the lower left quadrant double negative for FITC and PE. The right hand panel shows that >97% of cells were positive for SSEA4 (PE) and <0.5% positive for SSEA1 (FITC). Figure 3.8B shows the % of SSEA4 (stage-specific embryonic antigen 4) and SSEA1 (stage-specific embryonic antigen 1) positive cells after each subsequent 5 passages. SSEA4 is a cell surface marker present on all undifferentiated hPSC, whereas SSEA1 is only present on differentiated cells. These markers have been routinely used as positive and negative markers of pluripotency in hPSC since their initial isolation (Thomson *et al*, 1998). As can be seen, consistent enzymatic passage supported by either T16 or Y27632 has no effect on the expression level of the pluripotency marker SSEA4, with both remaining consistent with >90% positive cells. Furthermore the differentiation marker SSEA1 remains consistently low (expressed on <5% of cells). This shows that T16 maintains a cell surface marker profile consistent with that expected of hPSC, and comparable to that achieved with Y27632 treatment.

3.3.3 Analysis of pluripotency markers by immunocytochemistry

In order to further analyse the expression of pluripotency markers after prolonged treatment with T16 or Y27632, ICC was performed. Cells were fixed and stained at passage one and passage 30 as described in section 2.4.10. The use of T16 or Y27632 did not influence the number of cells expressing the key pluripotency related transcription factor Oct4 or the commonly used pluripotency marker Tra-1-81 even after 30 consecutive passages (figure 3.9). Again this suggests that T16 treatment does not have a detrimental effect on expression of pluripotency related proteins.

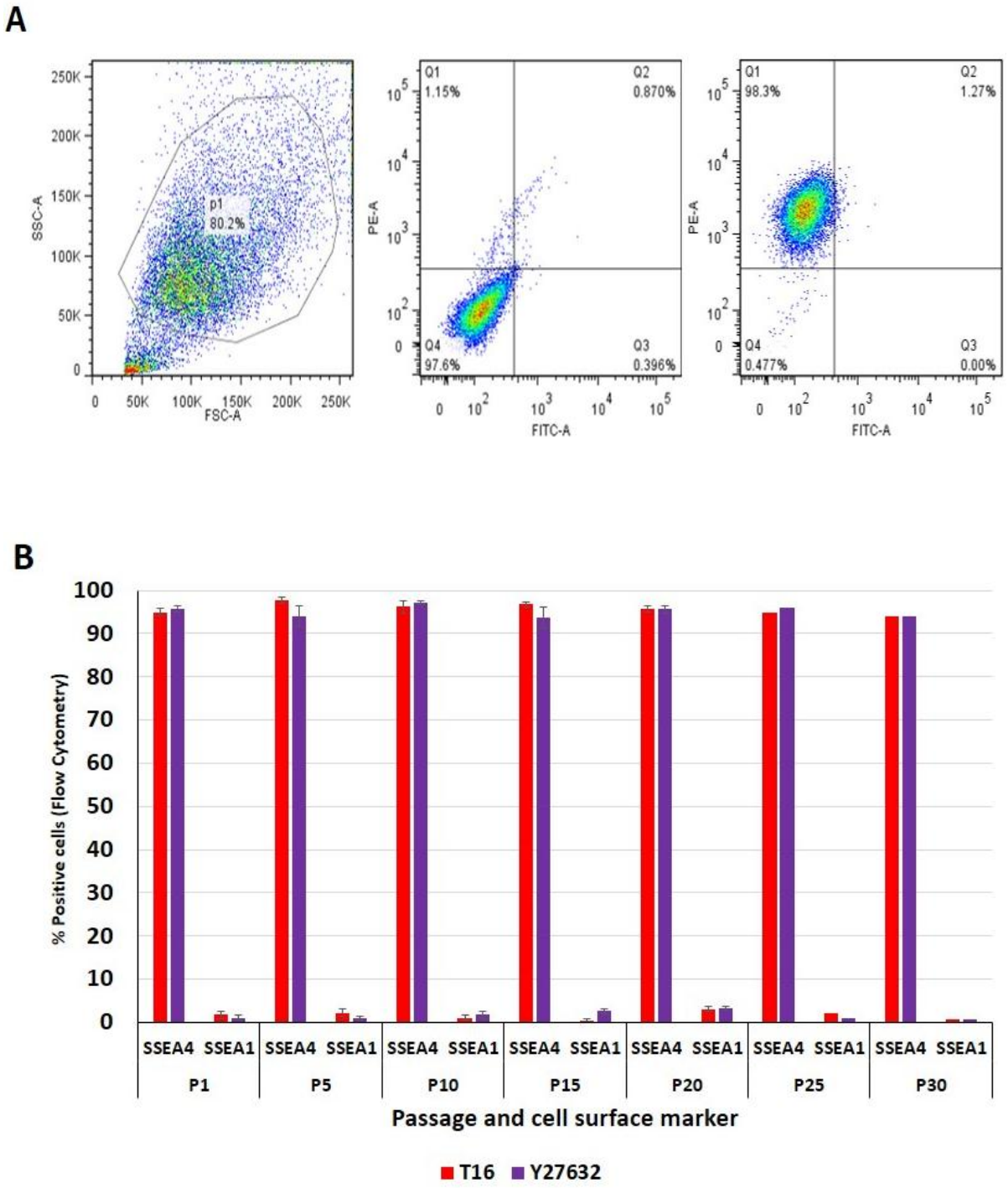


Figure 3.8- Flow cytometric analysis of hPSC surface markers. HPSC (H1 hESC) were enzymatically passaged using T16 or Y27632 for 30 consecutive passages. Cells were harvested at the point of passage and analysed via flow cytometry for the pluripotency marker SSEA4 and differentiation marker SSEA1. (A) Shows representative data produced, with the cell population imaged using forward and side scatter in the left hand panel, isotype controls in the centre panel and cells double stained for SSEA4 (PE) and SSEA1 (FITC) in the right hand panel. (B) Shows that both T16 and Y27632 treated cells maintained consistently high expression of SSEA4 over 30 consecutive passages, with negligible expression of SSEA1 being observed. Data shown as mean percentage of positive cells ±SEM (n=3) up to and including passage 20. Passages 25 and 30 represent values from an n=1.

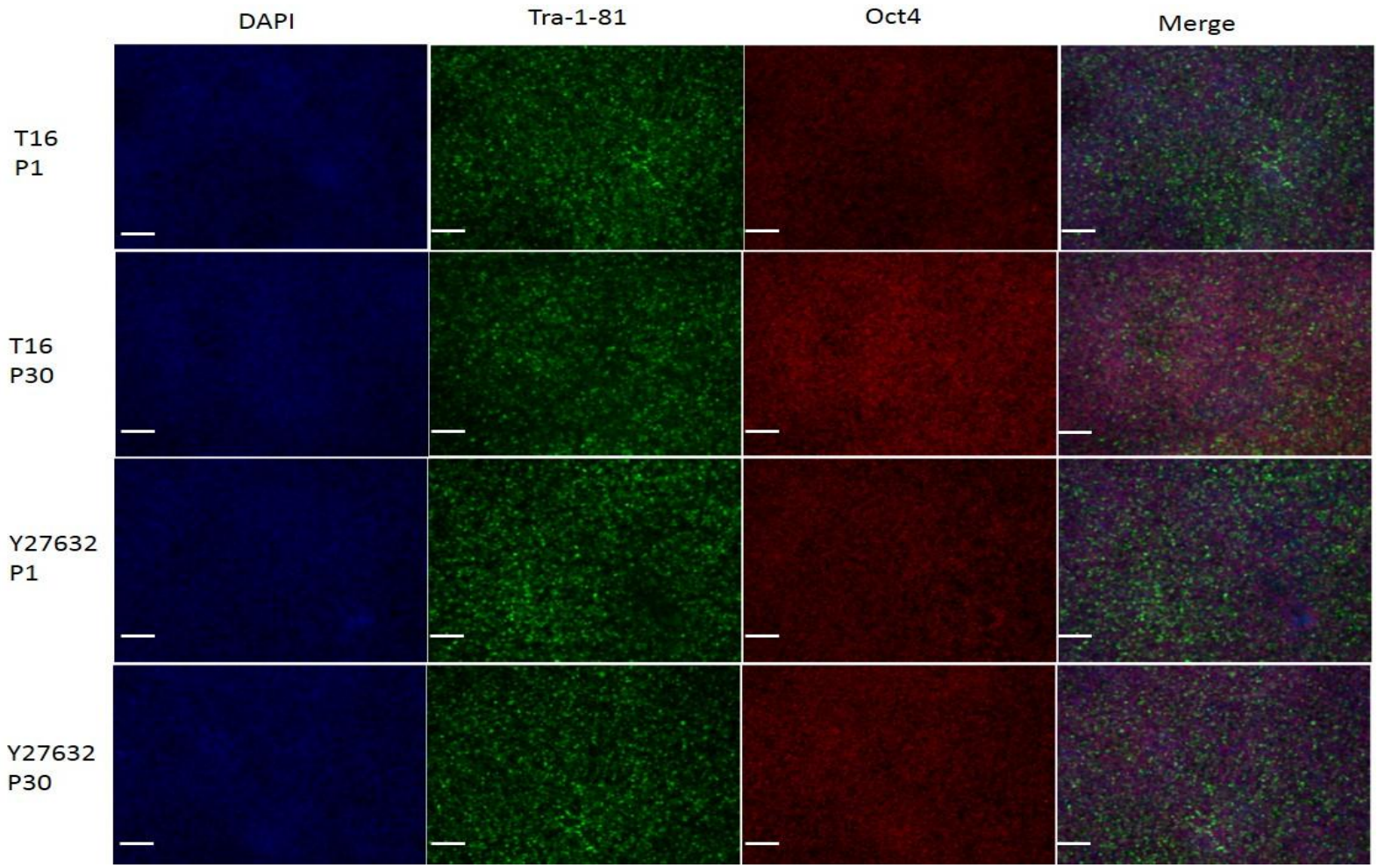


Figure 3.9- Expression of pluripotency markers. Cells passaged in T16 or Y27632 for 30 consecutive passages were fixed and stained for pluripotency markers Tra-1-81 (green) and Oct4 (red) and co-stained with DAPI (blue). Cells show maintained expression of these two markers over 30 passages, with the majority of cells staining double positive. Scale bar represents 100µm.

3.3.4 TaqMan qRT-PCR analysis of pluripotency and early differentiation markers

Although it has been established that treatment with T16 does not have any detrimental effect on the expression of key pluripotency markers such as Oct4 and SSEA4, the effect of prolonged exposure to either T16 or Y27632 on expression of a wider range of genes was still unknown. To investigate any alterations in gene expression, qRT-PCR was performed utilising the higher throughput TaqMan Low Density Array or TLDA platform. This platform utilises micro fluidic plates capable of simultaneously performing 384 reactions without requiring large amounts of sample. The cards allow a maximum of 8 samples (including 48 reactions/sample) to be processed using only 9µl of cDNA per sample. RNA was isolated from T16 and Y27632 treated hPSC (H1 hESC) at passages 1, 5, 10 and 15 with cDNA being synthesised as described in section 2.3.3, and TLDA plates were processed as detailed in section 2.3.5. Genes analysed include the key transcription factors Oct4 (POU5F1), Nanog and SOX2, markers of 'stemness' as well as a range of differentiation markers. Figure 3.10 shows expression of these key pluripotency factors as well as the early differentiation markers GATA4, PAX6 and FOXA2. As can be seen there are no significant differences in expression of the core pluripotency transcription factors between drug treatment and passage number. Furthermore, there is no significant change in the relatively low expression of GATA4 (mesoderm), PAX6 (ectoderm) or FOXA2 (endoderm). As expected, expression of these genes (as well as other differentiation related genes) remains low, showing that cells were not undergoing differentiation.

A table detailing the results for all analysed genes can be seen in appendix 1 (Table A1).

Cumulatively, the data presented in section 3.2.5.1 through 3.2.5.3 show that multiple and consecutive passage using T16 to support survival does not alter the key markers of pluripotency or differentiation, suggesting that T16 can be used as a long term hPSC culture reagent and an alternative to Y27632.

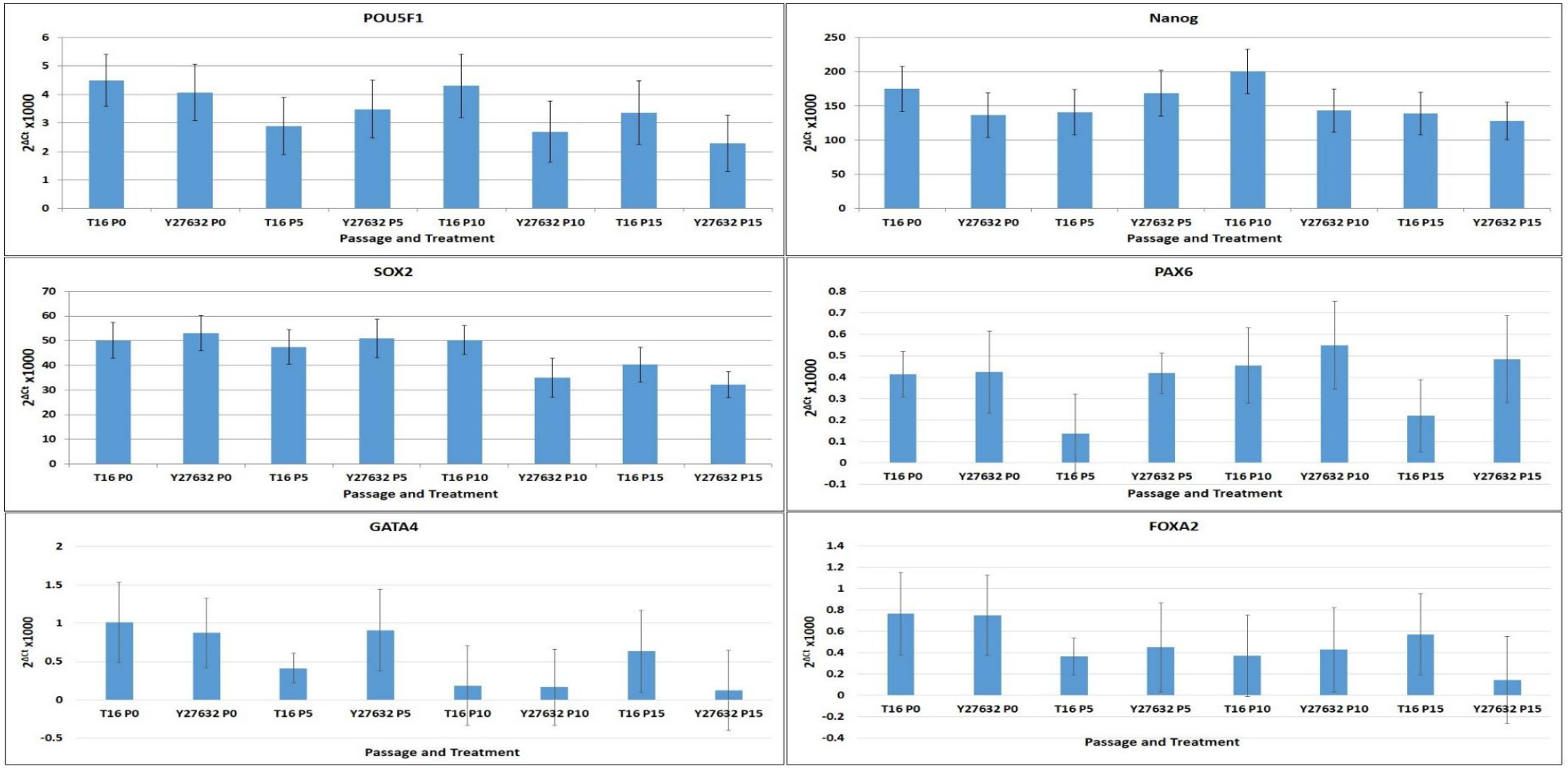


Figure 3.10- Relative expression of pluripotency and differentiation markers. H1 hESC cells treated with either T16 or Y27632 for 1, 5, 10 or 15 passages had mRNA expression analysed via qRT-PCR based TLDA assays. Data shows that the pluripotency markers POU5F1 (Oct4), Nanog, and SOX2 maintain steady expression over the 15 passages. Furthermore, differentiation markers PAX6, GATA4 and FOXA2 retain consistently low expression throughout the 15 passages. Data shown as mean $2^{\Delta Ct}$ x1000 ± SEM. Experiments were performed included 3 technical replicates, each with biological triplicates (n=3 in triplicate).

3.3.5 Treatment with T16 does not affect the pluripotent potential of hPSC

HPSC (hESC cell line H1) that had been enzymatically passaged with either T16 or Y27632 for 30 consecutive passages were passively differentiated alongside mechanically maintained cells using a mixture of embryoid body based suspension culture and adherent culture as described in section 2.2.1.7. Differentiated cells were subsequently fixed and stained for markers from each of the three germ layers. Long term exposure to either survival compound did not block the differentiation towards mesoderm (smooth muscle actin; SMA), endoderm (alpha fetoprotein; AFP) or ectoderm (paired box protein; PAX6). As expected, untreated mechanically passaged control cells, included as a positive control, were also able to differentiate into each of the three germ layers (figure 3.11).

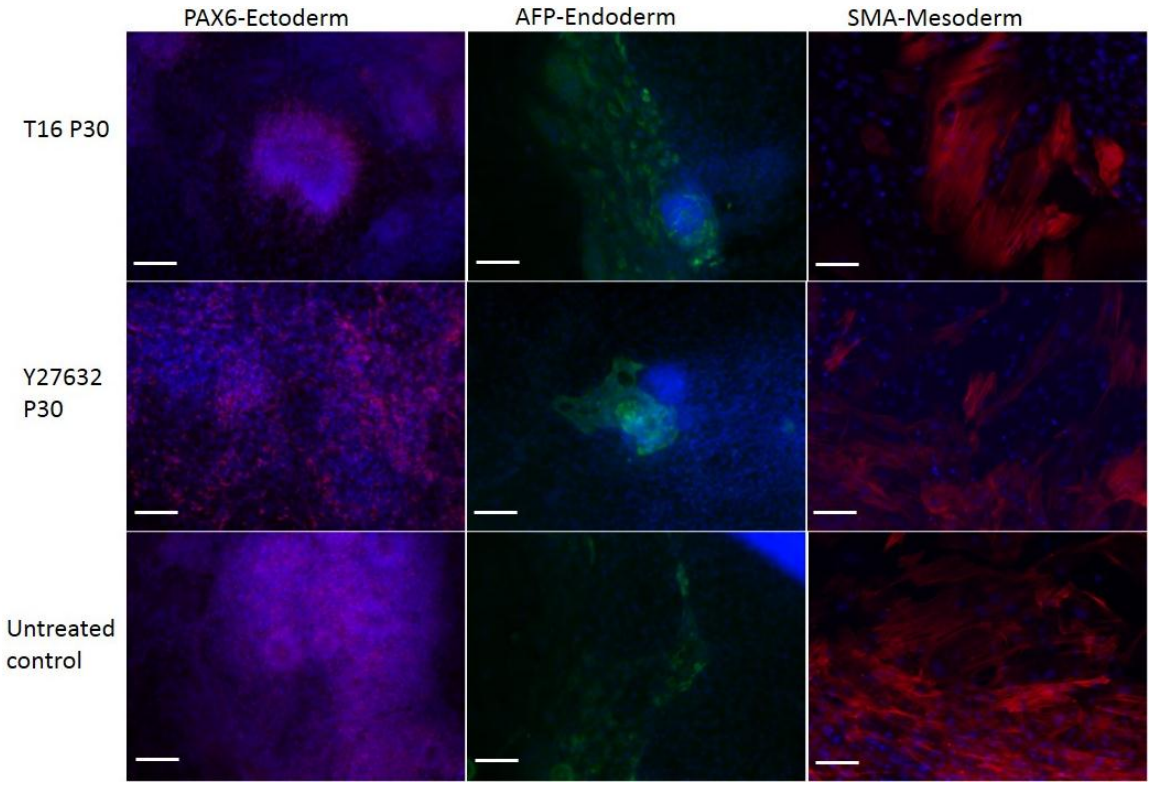


Figure 3.11- Passive differentiation of hPSC after T16 treatment. HPSC (h1 hESC) treated for 30 passages with T16 or Y27632 were passively differentiated for 2 weeks in suspension culture, followed by 2weeks adherent culture, fixed and stained for markers of the three germ layers. Control cells that had been mechanically passaged and unexposed to any survival compound were also differentiated and stained in the same way. Pax6 is stained red in the left hand column, AFP Green in the centre column and SMA red in the right hand column. All cells were co-stained with DAPI in Blue. Scale bar represents 100µm.

3.3.6 T16 does not prevent directed differentiation

Previous results indicate that the use of either survival compound does not prevent the passive differentiation of hPSC into any of the three germ layers. To further investigate the potential effect on differentiation, the capacity of treated hPSC to undergo directed differentiation was also investigated. Cells (hiPSC, NMF-iPS6) that were treated with T16 or Y27632 for 20 consecutive passages were induced to differentiate towards the haematopoietic cell lineage using a feeder-free suspension based differentiation protocol (Mountford lab, patent filed). As a control, untreated cells that had been maintained using mechanical passage were differentiated alongside T16 and Y27632 treated cells. Flow cytometric analysis of haematopoietic markers such as CD34 (~40% positive cells) at day 10, as well as the more mature erythroid markers GlyA and CD71 at day 24 (>90% of cells positive) confirmed the capacity of cells to differentiate towards erythrocytes (figure 3.12A). Notably, the marker profile was unchanged between treatment type. Furthermore, the cumulative expansion over the 24days of differentiation (figure 3.12B) shows a greatly reduced expansion in Y27632 treated cells when compared to either T16 treated or control cells. This supports unpublished data (Mountford lab) suggesting that Y27632 treatment reduces the proliferative potential of hPSC differentiated towards RBC after Y27632 use raising concerns over the utility of Y27632 for large scale applications. Critically, data here show that T16 does not share this negative effect and further enhances the commercial usefulness of T16.

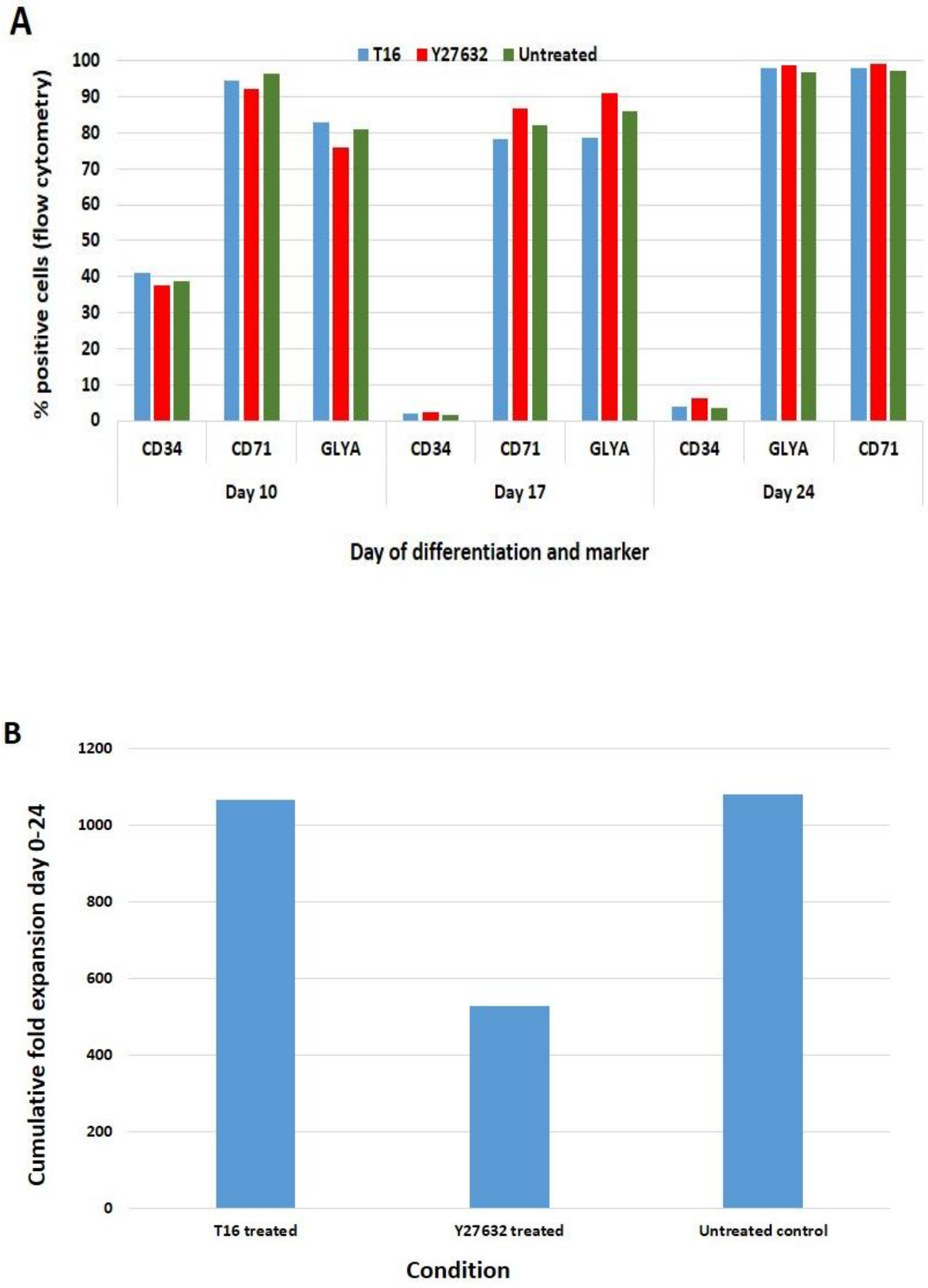


Figure 3.12- Directed differentiation of hPSC. HiPSC (NMF-iPS6) treated for 20 consecutive passages were differentiated towards RBC. (A) Cells were ~40% positive for CD34 at day 10 and >90% for GlyA and CD71 at day 24 regardless of treatment type. (B) The cumulative expansion of cells shows that Y27632 treated cells did not expand as well as T16 or untreated cells over the 24days. Data shown from n=1.

3.4 T16 can be used transiently or as an additive to culture medium

3.4.1 Cell survival

To determine whether T16 could be added to culture medium as a permanent addition, allowing hPSC to be enzymatically passaged whenever necessary without pre-treatment, cells were treated in parallel either transiently as described above or with T16 or Y27632 included continuously i.e. included in basal medium during daily feeding. Cells were passaged at 100% confluence as described above, which was approximately every 4-5 days, with the experiment lasting approximately 12 weeks. As can be seen in figure 3.13, cells treated continuously with T16 had survival comparable to that seen when cells were treated transiently. This was largely the case with Y27632 treatment; however, there were numerous passages where survival was lower than expected in the continuously treated cells. This was particularly evident between passages 10-12 when survival was consistently lower than transiently treated cells. There were no differences thereafter which may be indicative of culture adaptation or the acquirement of chromosomal abnormalities.

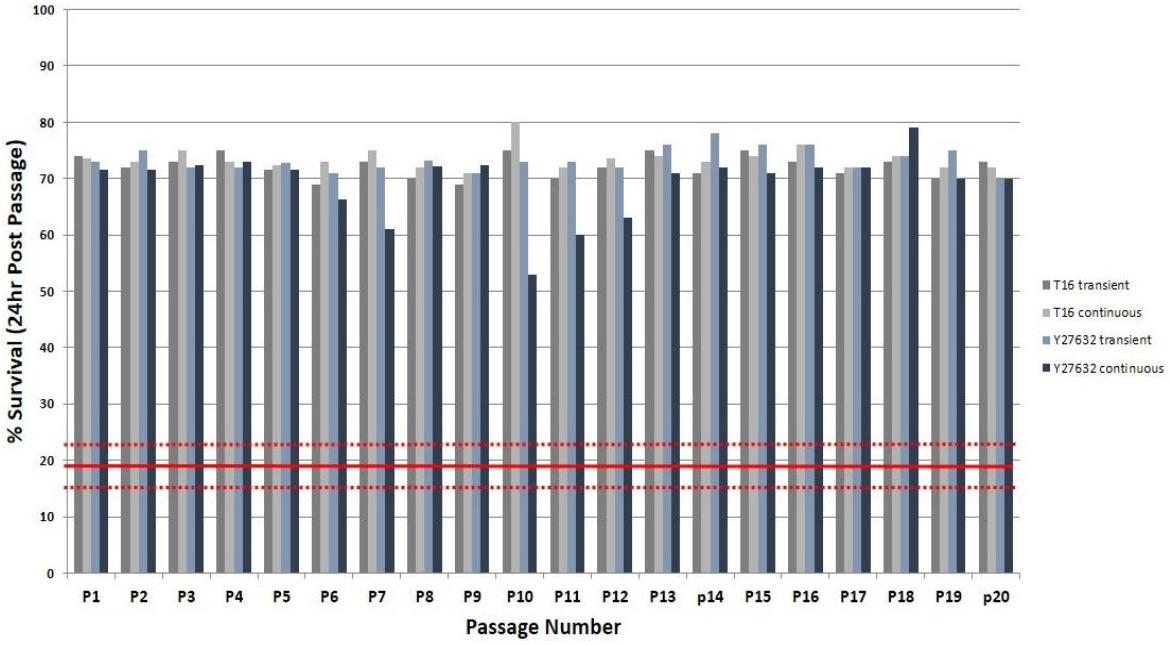


Figure 3.13- Continuous versus transient use of survival compounds. HiPSC (NMF-iPS6) were treated either transiently or continuously with T16 or Y27632 and cell survival assayed. HiPSC survival appears unchanged by transient or continuous use of T16, whereas there is a slight dip around P10 in cells treated continuously with Y27632. This data represents n=1. Red bars represent the average survival of untreated cells ±SEM, n=3.

3.4.2 Maintenance of pluripotency

As with previous experiments testing the effect of transient use of T16 and Y27632 over 30 passages of H1 hESC, it was critical to confirm that continuous use of T16 did not impact negatively on the expression of pluripotency and differentiation related markers. Cells (hiPSC, NMFiPS6) treated either continuously or transiently with T16 or Y27632 expressed consistently high levels of pluripotency marker SSEA4 and low expression of the differentiation marker SSEA1 over 20 consecutive passages as determined by flow cytometry (figure 3.14). This confirms that continuous use of T16 has no detrimental effect on the cell surface marker profile of hPSC.

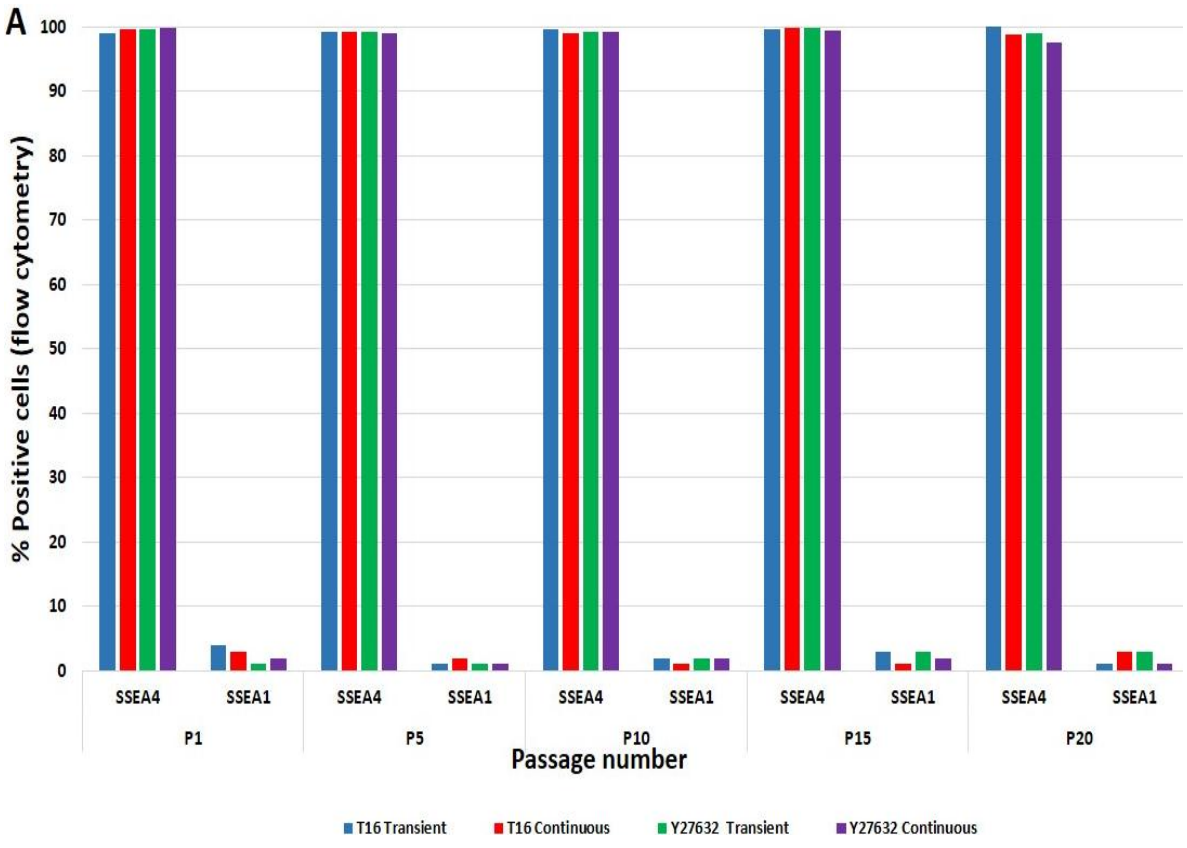


Figure 3.14- Expression of pluripotency markers after continuous exposure to T16 or Y27632. HiPSC (NMF-iPS6) treated either transiently or continuously with T16 or Y27632 were analysed at passage 1, 5, 10, 15 and 20 for the pluripotency marker SSEA4 and differentiation marker SSEA1. Continuous treatment with T16 or Y27632 had no negative effect on the expression of SSEA4 with all passages tested having greater than 95% positive cells. Similarly, the expression of SSEA1 was unchanged.

3.4.3 Karyotypic stability of continuously treated cells

HPSC that had been treated for 20 consecutive passages either continuously or transiently with both T16 and Y27632 were also analysed for karyotypic stability. Cells treated with T16 were normal (46 XY) after continuous and transient treatments, however Y27632 treated cells were not. Unlike the experiment shown in Section3.3.1 (figure 3.7) above in which H1 hESC were used, in this case hiPSC treated either continuously or transiently with Y27632 has an additional copy of chromosome 12 in 2 of 20 cells and 1 of 20 cells analysed respectively (Figure 3.15). Although these results are only from a single experiment, all four conditions were initiated from the same well of normal hiPSC, which may suggest that T16 is better suited for supporting stable expansion of hPSC after enzymatic disaggregation. There may also be differnces in stability between hESC and hiPSC lines.

3.4.4 Long term treatment with T16 does not affect the pluripotent potential of hPSC

The inclusion of T16 in culture medium continuously for 20 passages did not negatively affect the differentiation into any of the three germ layers when cells were allowed to undergo passive differentiation as described previously. Figure 3.15 shows cells treated continuously with either T16 or Y27632 were able to passively differentiate into ectoderm (PAX6), endoderm (AFP) and mesoderm (SMA).

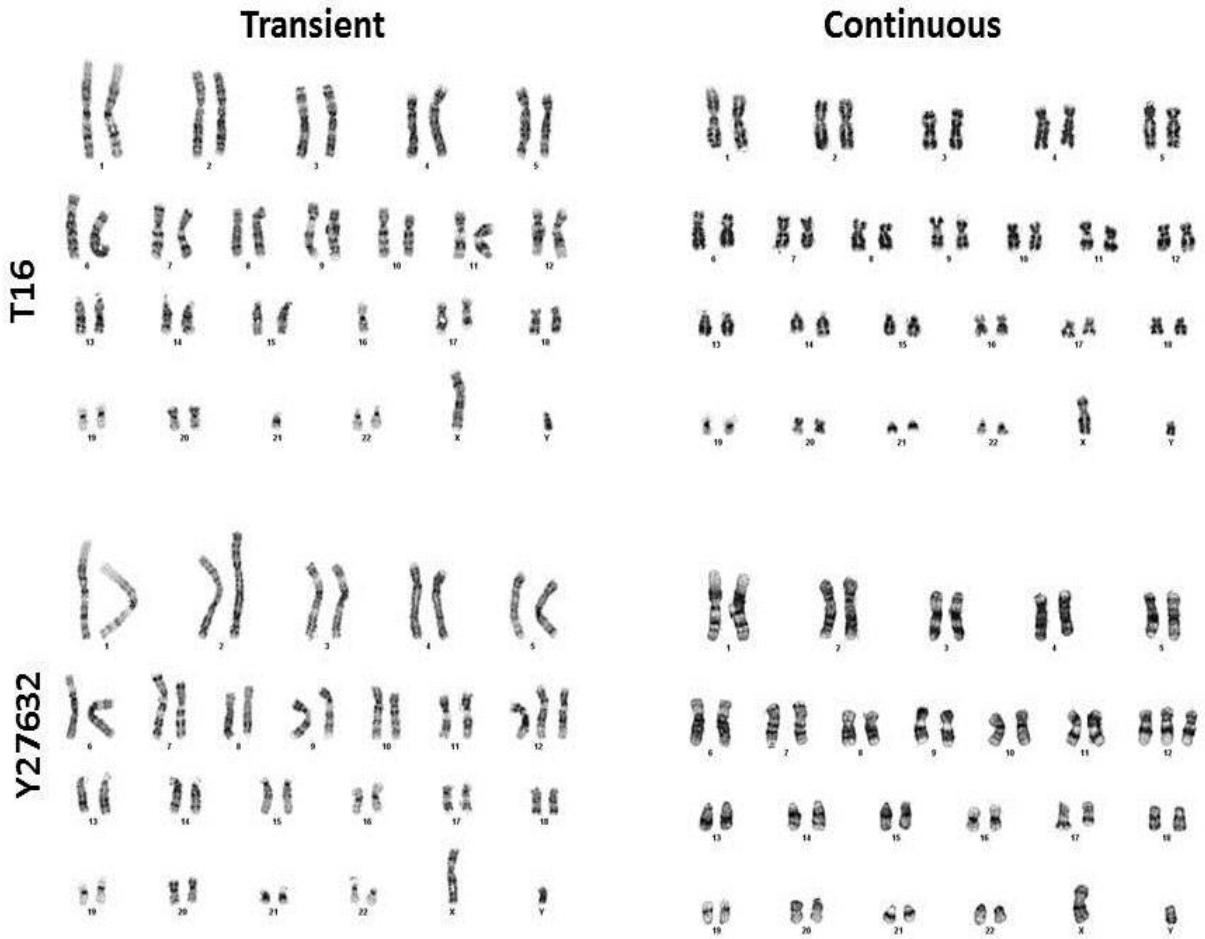


Figure 3.15- Karyotypic stability of continuously and transiently treated hiPSC. Cells (NMF-iPS6) treated either continuously or transiently with T16 or Y27632 were analysed for chromosomal abnormalities. T16 cells were normal (46 XY) regardless of treatment type. Y27632 treated cells had +12 in 1 out of 20 and 2 out of 20 when used transiently and continuously respectively.

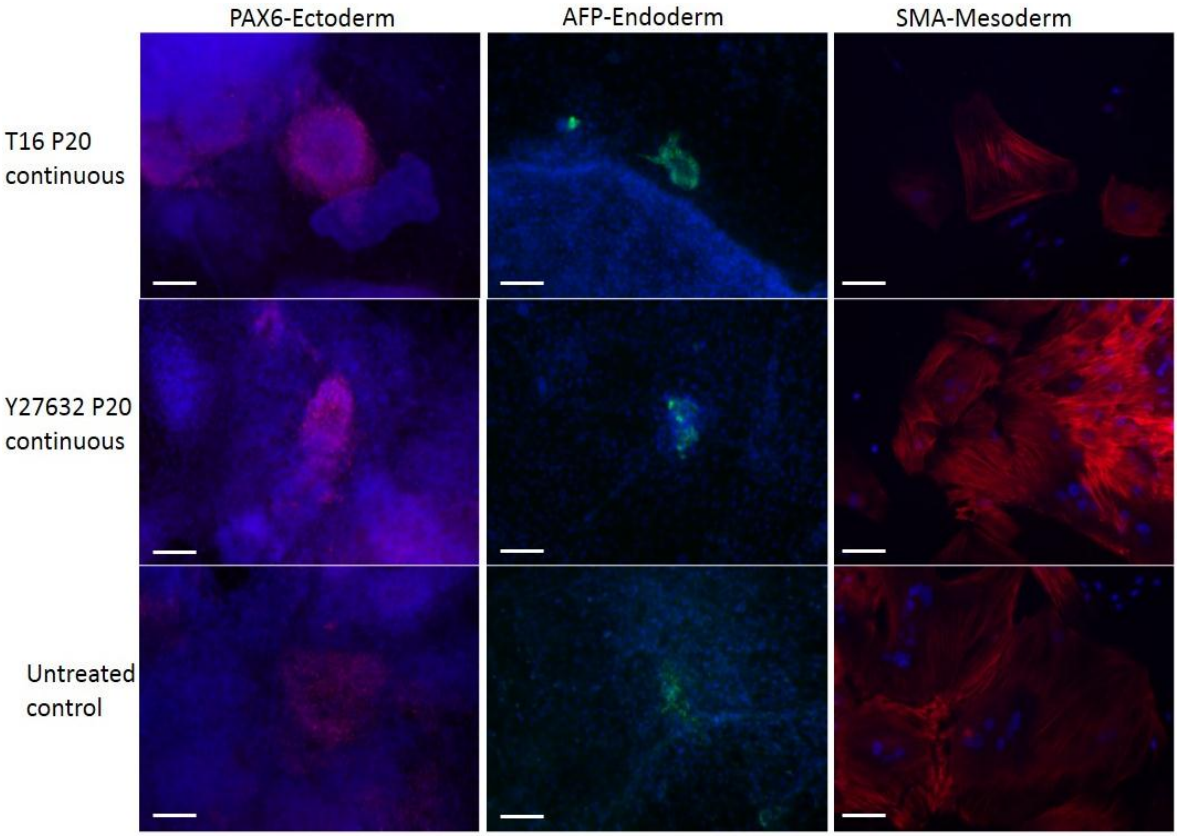


Figure 3.16- Passive differentiation of hPSC treated continuously with survival compounds. HiPSC (NMF-iPS6) that had been cultured for 20 consecutive passages in the continued presence of T16 or Y27632, were passively differentiated and stained for markers of the three germ layers alongside untreated mechanically passaged control cells. PAX6 (ectoderm) staining red in the left hand column, AFP (endoderm) staining green in the centre column and SMA (mesoderm) staining red in the left hand column, positive cells were seen in all three conditions. Scale bar represents 100µm.

The data presented in this sections 3.2.7.1 to 3.2.7.4 show that T16 can be used either transiently at the point of passage, or can be added continuously to Stempro SFM culture medium. Continuous use supports similar survival, marker expression and differentiation potential to transient use and offers the very interesting possibility that T16 could be included in basal hPSC media formulations to facilitate routine enzymatic passaging at will.

3.5 Summary

Presented within this chapter are data which further characterise the hPSC survival compound T16. The results show that T16 can be used in a number of culture mediums including completely undefined MEF conditioned medium and more chemically defined culture mediums such as StemPro SFM or mTeSR. Furthermore, T16 produces the same survival effect regardless of the ECM component used to facilitate adhesion. The response to T16 is not cell line specific; with the pro-survival effect being reproduced in 4 lines tested which include both hESC and hiPSC. T16 treatment results in cell survival equivalent to that of the commonly used ROCK inhibitor Y27632 and supports hPSC propagation for at least 30 consecutive passages without causing karyotypic instability when used transiently, or for at least 20 consecutive passages when used continuously.

Furthermore, the use of T16 does not negatively affect the expression of pluripotency related genes at either the mRNA transcript level (qRT-PCR analysis) or the protein level (ICC and flow cytometry). In addition to this, a panel of differentiation related markers remained unchanged in response to T16 treatment after 15 consecutive passages using the compound as determined by qRT-PCR analysis.

These data also confirm that treatment with T16 either transiently or continuously has no effect on the differentiation capacity of hPSC which passively differentiated into derivatives of all three germ layers or successfully underwent directed differentiation to red blood cells.

Taken together, the work detailed in this chapter suggests that T16 is at least as useful as Y27632 and may even prove to be more so given that it does not appear to have a negative impact on directed differentiation towards red blood cells. However, elucidating how T16 mediates its survival effect will be crucial in better understanding the survival/death mechanism in hPSC.

4 T16 pathway analysis: Potential for a RhoA independent survival axis

4.1 General Introduction

There have been a number of studies performed recently with the intention of finding additional PSC survival compounds, however, even when using a high-throughput approach this has been relatively unsuccessful. All recently identified compounds that have successfully supported enzymatic passage of hPSC have subsequently been shown to inhibit the ROCK signalling pathway (Andrews *et al*, 2010; Xu *et al*, 2010; Barbaric *et al*, 2010).

As previously stated, T16 was initially identified as a non-ROCK inhibitory hPSC survival compound (unpublished data). Having optimised the performance of T16, and shown that it supports enzymatic passage of hPSC to a comparable degree to the Rho Kinase inhibitor Y27632 without any detrimental effects, experimentation was undertaken to further elucidate the mode of action of T16.

The aims of these studies were to:

- Confirm the kinase inhibition profile of T16 at the optimal concentration of 30μM (previous screen data was generated using a concentration of 10μM).

- Investigate the potential role of any kinases showing altered activity in response to T16 treatment.

- Determine if T16 mediates its pro-survival effect via upstream or downstream regulation of the RhoA/ROCK/pMLC axis.

4.2 T16 is not a Rho kinase inhibitor

To reaffirm that T16 did not inhibit ROCK or the closely related PRK2, the compound was rescreened at the Dundee International Centre for Kinase Profiling at both the standard 10μM and optimum 30μM concentrations. This screen utilises a radioactive filter-binding assay (^{33}P-ATP) to assess kinase inhibition. Results confirmed that T16 had no inhibitory effect on ROCK2 at a concentration of 10μM (96% activity), and in fact slightly promoted the kinase activity of ROCK2 at the optimum concentration of 30μM (118% activity). T16 also had very limited effect on PRK2, showing 76% and 98% activity when used at 10μM and 30μM respectively (figure 4.1).

	% activity	
	10μM	30μM
ROCK2	96	118
PRK2	76	98

Figure 4.1-T16 does not inhibit Rho kinase. T16 kinase inhibition was assayed using a radioactive filter-binding assay at the Dundee International Centre for Kinase Profiling. The optimum concentration of T16 (30μM) did not inhibit either ROCK2 or PRK2.

4.2.1 T16 has minimal effect on *in vitro* protein kinase activity

From an expanded panel of 121 kinases, T16 only partially inhibited one particular kinase; receptor interacting protein kinase (RIPK2). The kinase inhibition profile of T16 shows 51% inhibition of RIPK2 when used at 10μM and 72% when used at 30μM. The highest level of inhibition other than that of RIPK2 was observed in EPH-B2 (ephrin type-B receptor 2) at 10μM (~45%). However at the optimum concentration of T16 this inhibition was reduced to only 3%, suggesting that EPH-B2 inhibition is unlikely to be the cause of T16 mediated cell survival. All other kinases were inhibited by <30% when tested at the optimum concentration of 30μM (Figure 4.2).

4.2.1.1 Potential role of RIPK2

RIPK2 is a serine/threonine kinase with a caspase recruitment domain (CARD) present in the c-terminal domain of the protein. RIPK2 has been reported to promote apoptosis via interactions with mediators of apoptosis such as CLARP (caspase-like apoptosis-regulatory protein) downstream of the death receptor CD95 (Inohara *et al*, 1998; Inohara *et al*, 1997). Given that RIPK2 was the only kinase that showed any level of inhibition in response to T16, and that it has a reported pro-apoptotic activity, the potential role of this protein in hPSC was investigated. Firstly expression of RIPK2 in hPSC was confirmed by qRT-PCR analysis. Untreated control cells as well as T16 and Y27632 treated hPSC expressed mRNA for RIPK2 the level of which was maintained after 3 consecutive enzymatic passages supported by either T16 or Y27632 (figure 4.3A). Although there was an increase in mRNA in Y27632 treated cells at passage 2 this was not significantly higher than any other measurement and was reduced by passage 3. Furthermore there were no significant differences in mRNA expression between treatment types suggesting T16 does not transcriptionally upregulate expression of RIPK2.

Efforts were made to confirm the expression of endogenous RIPK2 protein using antibodies against the unphosphorylated, inactive state as well as the phosphorylated (ser176) active state, however antibodies from 2 separate suppliers produced no signal.

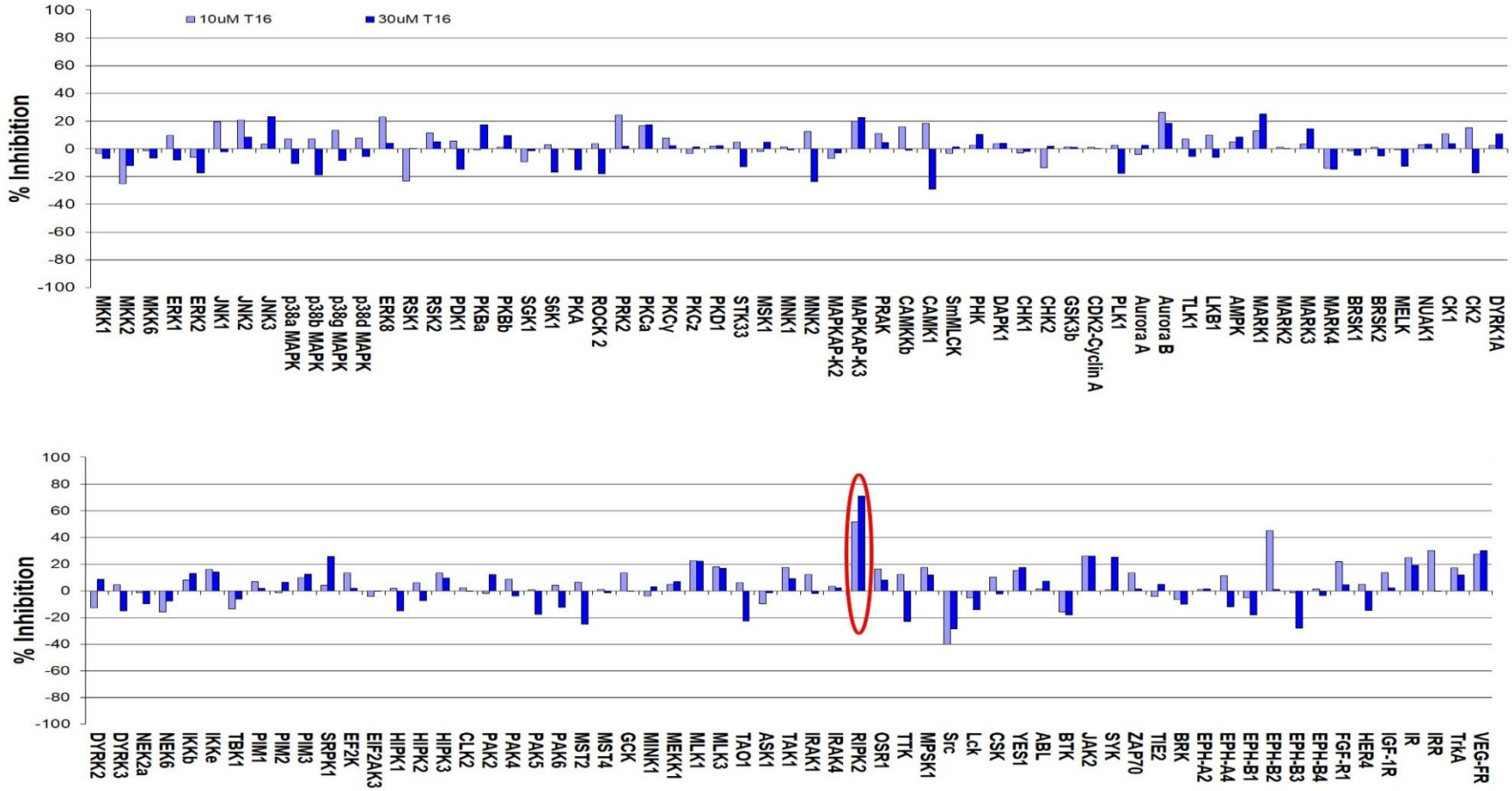


Figure 4.2- Kinase profile of T16 at 10μM and 30μM. Kinase inhibition on 121 kinases was assayed using a radioactive filter-binding assay at the Dundee International Centre for Kinase Profiling. T16 had only minor effects on all but one of the kinases tested. Receptor Interacting Protein Kinase 2 (RIPK2) (circled in red), which was inhibited by 51% and 72% when T16 was used at 10μM or 30μM respectively.

RIPK2 has been documented to have key roles in cells of both the innate and adaptive immune systems and has been shown to exert its effect via downstream activation of NFκB signalling (Hasegawa et al, 2008). NFκB is activated by IκB kinase (IκK)-mediated release of the IκB inhibitory subunit, therefore inhibition of IκK and the resulting suppression of NFκB might recapitulate the effect of inhibiting RIPK2. If T16 mediates its effect via inhibition of RIPK2 and the downstream NFκB pathway, inhibition of IκK may recapitulate this effect. A potent inhibitor of IκK; TPCA-1 was therefore used either alone or in combination with T16 or Y27632 treated hPSC (as described in sections 2.2.1.2 and 2.2.1.4) to test the effect on survival and to test whether inhibition of NFκB signalling abrogates the effect of T16. Inhibition of NFκB signalling had no effect on hPSC survival, as untreated control cells (no survival compound) + TPCA-1 did not show significantly higher survival when compared to DMSO controls. Similarly the inclusion of TPCA with T16 or Y27632 did not ameliorate the increase in survival seen in response to either compound (P=<0.001). These data also show that inhibition of IκK, and thus NFκB activity, by TPCA-1 did not impact hPSC survival *per se* or the pro-survival effect of T16 or Y27632 (figure 4.3B).

As inhibition of signalling events downstream of RIPK2 activation had no effect on hPSC survival, this suggests that inhibition of RIPK2 is not the mode of action of T16. Also as T16 only partially inhibited RIPK2 at the effective dose and given the paucity of available resources to target this pathway, further investigations were not pursued at this time.

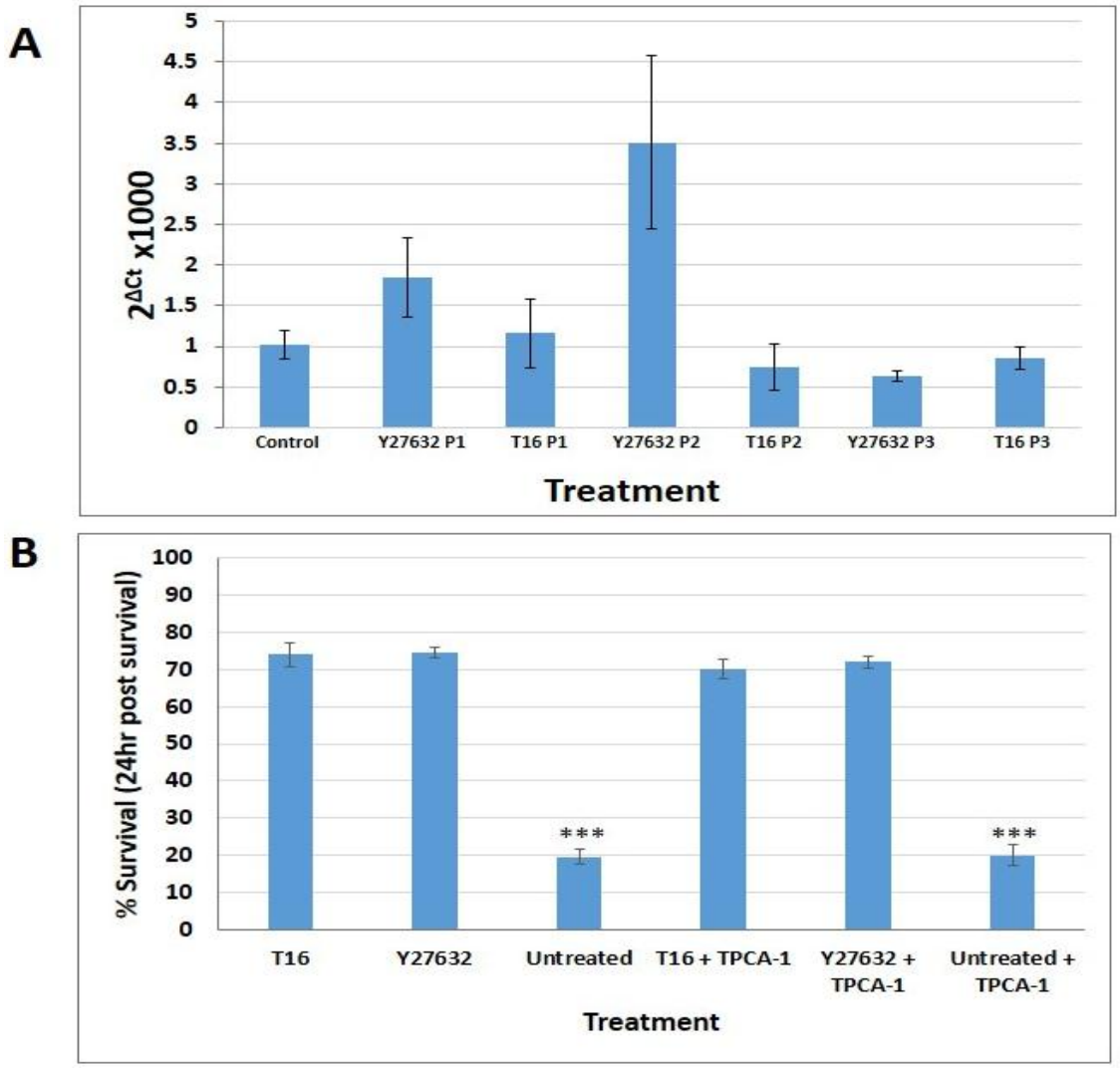


Figure 4.3- qRT-PCR analysis of RIPK2 and downstream signalling inhibition. Cells (H1 hESC) treated with T16, Y27632 as well as untreated control cells were analysed for expression of RIPK2. RNA was isolated from cells at the point of passage (after the various treatments). Cells show steady expression independent of treatment type or length (A). Cells were treated with 10μM of the potent IκK inhibitor TPCA-1 in the presence or absence of T16 or Y27632. Inhibition of IκK had no effect on cell survival, with untreated control cells + TPCA-1 showing no significant increase in cell survival compared to the untreated control. There was no significant difference in cell survival in T16 or Y27632 treated ± TPCA-1. T16 and Y27632 treated cells ± TPCA-1 had significantly higher cell survival than Untreated ± TPCA-1 (P=<0.001). Data shown as mean values ± SEM, n=3.

4.2.2 T16 does not inhibit the hyperphosphorylation of myosin light chain

It has been suggested that the cause of dissociation induced apoptosis of hPSC is the hyperphosphorylation of myosin light chain (MLC) and that the protective activity of Y27632 is to prevent this phosphorylation (Chen *et al*, 2010; Ohgushi *et al*, 2010 and Walker *et al*, 2010), therefore western blot analysis was performed to test whether T16 shares this biochemical effect. The data shown in figure 4.4 show that as expected the untreated cells had significantly higher levels of pMLC 15mins post-dissociation and that Y27632 blocked the increase (P=<0.01), however cells treated with T16 showed high levels of pMLC equivalent to those in untreated cells (P=>0.05). The higher levels of pMLC were maintained in untreated and T16 treated cells compared to Y27632 treated cells at later time points with T16 vs Y27632 being significantly different at 30mins (P=<0.05), 45mins (P=<0.05) and 1hr (P=<0.05). Although untreated cells did not reach significance versus Y27632 treated cells at these subsequent time points, the trend was towards increased levels of pMLC in untreated hPSC.

These data from untreated and Y27632 treated cells are consistent with those observed by others (Chen *et* al, 2010; Ohgushi *et* al, 2010), confirming that in response to dissociation there is a sudden increase in phosphorylated MLC and that treatment with Y27632 is able to prevent this hyperphosphorylation. Importantly though, treatment with T16 did not inhibit the phosphorylation of MLC strongly suggesting that T16 does not share the same mechanistic downstream target reported for Y27632. These findings also clearly uncouple the inhibition of MLC hyperphosphorylation and hPSC cell survival supporting the hypothesis that there is an additional novel pro-survival pathway in these cells that may be elucidated using T16.

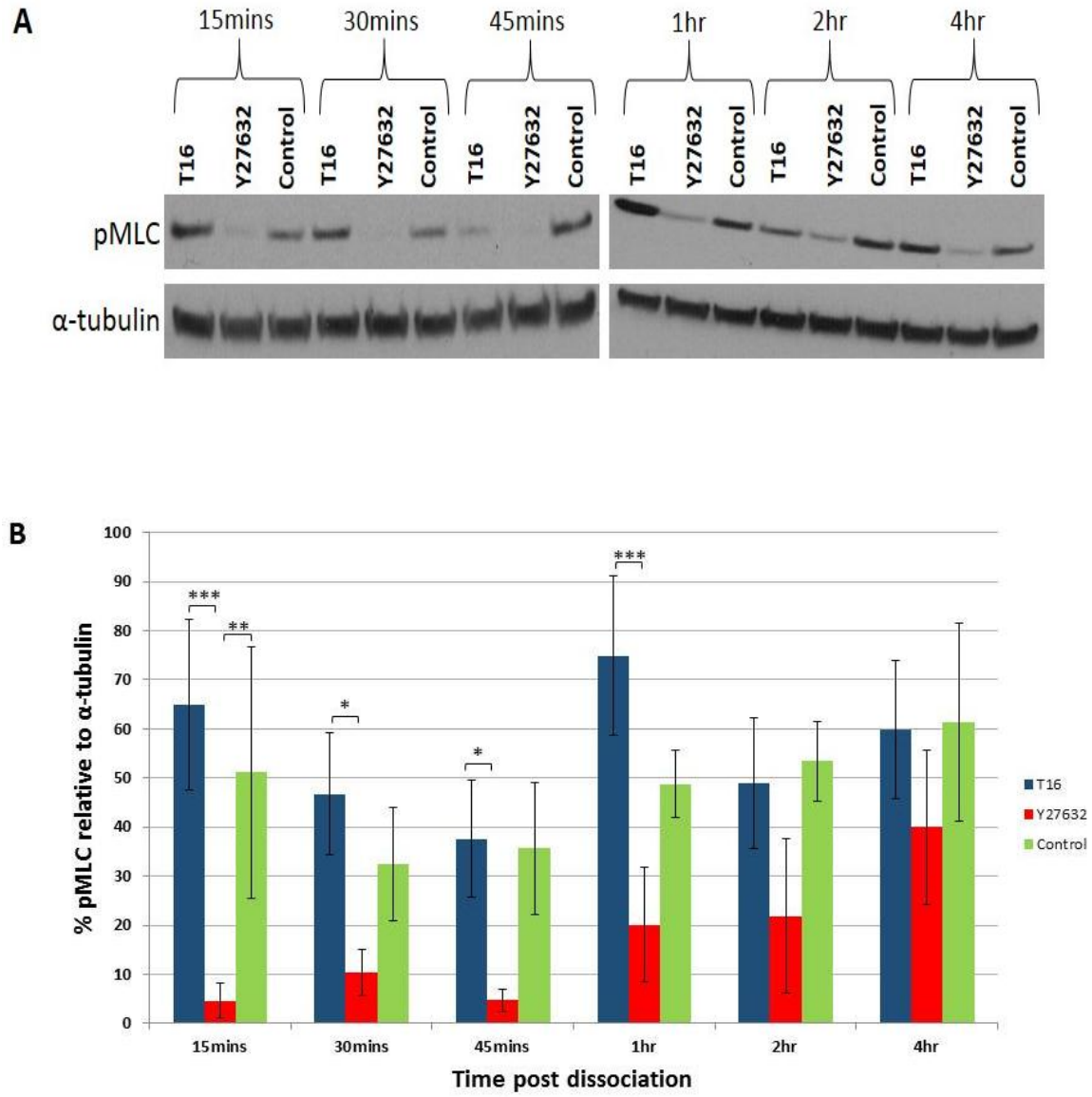


Figure 4.4- Analysis of levels of pMLC after treatment and dissociation of hPSC. Protein samples were taken from cells (NMF-iPS6) that were treated with T16, Y27632 or had no treatment (control) before being dissociated. Samples were harvested 15mins, 30mins, 45mins, 1hr, 2hr and 4hr post dissociation. Western blot analysis was performed. (A) Shows representative immunoblots for pMLC and α-tubulin. (B) Expression of pMLC relative to α-tubulin analysed via densitometry. Both T16 treated and untreated cells had significantly higher levels of pMLC 15mins post dissociation when compared to Y27632 treated cells (P=<0.001 and P=<0.01 respectively). T16 treated cells had significantly higher levels of pMLC when compared to Y27632 treated cells after 30mins (P=<0.05), 45mins (P=<0.05) and 1hr (P=<0.001). Data shown as mean ± SEM, n=3.

4.2.3 T16 does not inhibit RhoA, the upstream regulator of ROCK

Although it has been shown that T16 does not inhibit ROCK, it was possible that T16 was mediating survival by targeting the same pathway but acting upstream of ROCK. ROCK is a key effector of the small GTPase RhoA, therefore experiments were undertaken to test whether T16 inhibited RhoA activation. G-LISA assays, which measure levels of GTP bound (active) RhoA, were performed as described in section 2.4.6 with the percentage of GTP bound RhoA obtained by expressing the relative luminescence unit (RLU) value of samples relative to the RLU value of the RhoA control sample.

It has been reported by others (Ohgushi *et al*, 2010) that in response to dissociation there is a sudden increase in levels of GTP bound RhoA in hPSC. In order to validate the assay, untreated (DMSO only) hPSC were analysed alongside cells treated with the RhoA activator LPA and the RhoA inhibitor C3 (24hr pre-treatment). Cells were lysed directly on the plate to determine basal levels of GTP bound RhoA (0min), or dissociated and replated onto fibronectin coated plates for 30minuts before being harvested. As expected, there was a significant increase in GTP bound RhoA in untreated cells 30mins post dissociation when compared to the 0min samples ($p=<0.05$). Inhibition of RhoA with C3 prevented any significant rise in GTP bound RhoA 30minutes post dissociation compared to the 0min sample ($P=>0.05$), and LPA effectively induced RhoA activation with 0min samples having significantly higher levels of GTP bound RhoA ($P=<0.05$) than those treated with C3 or DMSO alone (figure 4.5A).

Having validated the assay, the effect on T16 and Y27632 treatment on GTP activation was investigated using both hiPSC and the hESC line H1. The experiment was performed as above with samples being harvested at 0mins, 15mins, 30mins and 45mins post dissociation.

When hiPSC were used, all three conditions had similar baseline levels of active RhoA prior to dissociation (0mins). Furthermore, all treatment types resulted in a general trend towards increased RhoA activity after dissociation. T16 and untreated cells had significantly increased levels of GTP bound RhoA 15mins, 30mins and 45mins post dissociation when compared to the time 0 sample

(P=<0.05) (figure 4.5B), with Y27632 treated cells similarly showing increased levels of GTP bound RhoA at each time point, although only reaching significance at 30mins and 45mins when compared to the time 0 samples (P=<0.05).

The repeated experiment with hESC showed a similar pattern to that seen in hiPSC, with samples 30mins and 45mins post dissociation having significantly higher levels of GTP bound RhoA versus the time 0 samples regardless of treatment (P=<0.05) (figure 4.5C). The similarity between cell types suggests that this response is not cell type specific and therefore likely to be biologically relevant.

These data are consistent with the literature, confirming that dissociation resulted in a sudden increase in GTP bound RhoA in hPSC. However, these findings expand on this and demonstrate that treatment with either T16 or Y27632 is unable to prevent this activation. These data support the hypothesis that T16 does not mediate its effect by inhibiting RhoA activation, and provides further evidence that there is a RhoA/ROCK independent mechanism of hPSC survival.

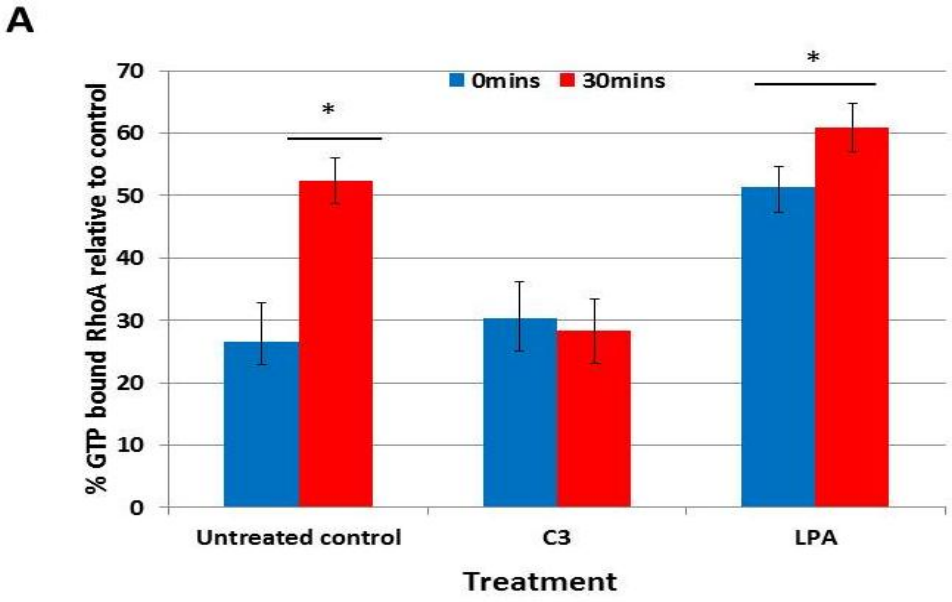


Figure 4.5- Continued overleaf

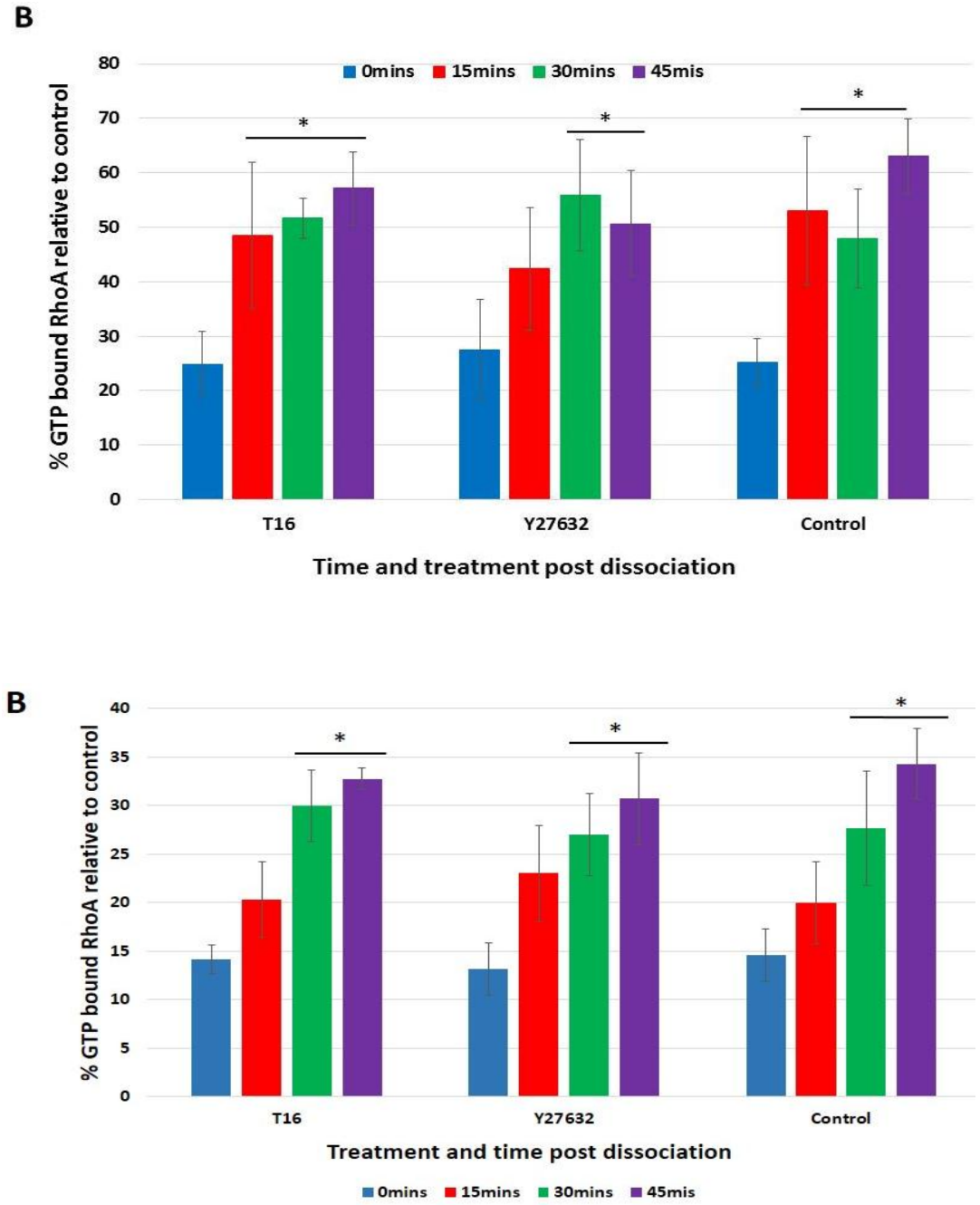


Figure 4.5- Levels of GTP bound RhoA in response to dissociation. G-LISA assays were performed on hPSC to assess the levels of GTP bound RhoA in response to dissociation. (A) Untreated control cells and the RhoA activator LPA and inhibitor C3 were used to validate the assay. Dissociation of untreated cells led to an increase in GTP bound RhoA when compared to the 0min samples (P=<0.05). The assay confirmed the capacity of C3 and LPA to inhibit and induce RhoA activation respectively. (B) Treatment with either survival compound was unable to prevent the increased activation of RhoA in hiPSC (NMF-iPS6) in response to dissociation, with increased GTP bound RhoA being observed 15, 30 and 45mins post dissociation in T16 treated cells (P=<0.05), and 30 and 45mins post dissociation in Y27632 treated cells (P=<0.05) when compared to time 0 samples. (C) Similarly, in the hESC line H1 there was a significant increase in GTP bound RhoA regardless of treatment type in 30min and 45min post dissociation samples compared to time 0 samples (P=<0.05).

4.2.3.1 Inhibition or forced activation of RhoA has no effect on T16 mediated cell survival.

Having shown that the RhoA inhibitor C3 and activator LPA, have the expected effect on RhoA activation status, (figure 4.5A), the effect on cell survival when used on hiPSC either alone or in combination with T16 was investigated. Cells were treated as described previously, with the inclusion of LPA and C3 at the same point as T16 (24 hours prior to dissociation) to maximise the effect. Cells were dissociated and replated in the presence of T16 ± C3 or LPA and cell survival was assessed 24 hours post dissociation. As expected, treatment with C3 alone leads to significantly increased cell survival versus untreated control cells (P=<0.001) (figure 4.6A). This can be attributed to its downstream effect on ROCK activation (inhibition of RhoA leads to a decrease in activation of ROCK which subsequently leads to a decrease in myosin hyperphosphorylation). The addition of C3 to T16 treated hPSC had no effect on cell survival which might be expected since both treatments appear to have beneficial effect on cell survival.

Further experimentation utilised the RhoA activator LPA and showed that treatment of hPSC with 5μM, 10μM or 20μM of LPA alone had no effect on cell survival (figure 4.6B), with all doses of LPA resulting in similar levels of survival to those seen in untreated cells. Also, when LPA was used in combination with T16 or Y27632, there was no effect on the pro-survival effect of either molecule (P=>0.05) at any dose of LPA.

These data suggests that T16 is able to support hPSC survival even in the presence of active RhoA which further supports a non RhoA dependent mechanism of survival.

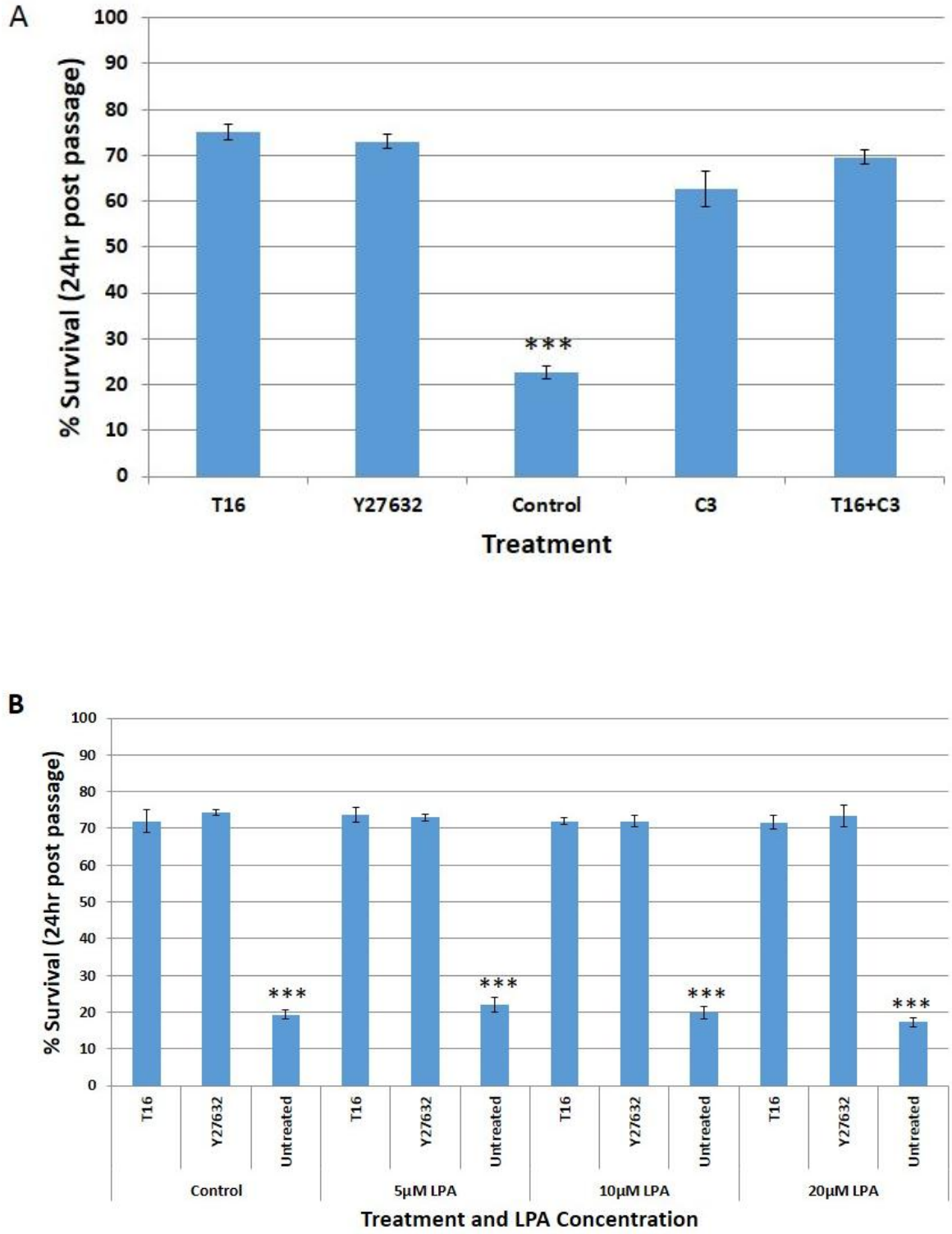


Figure 4.6- Effect of C3 and LPA treatment on survival. (A) HiPSC (NMF-iPS6) were treated with T16, Y27632, C3, C3 + T16 or left untreated, before being dissociated and replated. Survival was assayed 24hrs post passage. C3 treatment alone was sufficient to significantly increase cell survival compared to untreated control cells (P=<0.001). (B) A combination of T16 and C3 treatments had no significant effect on hiPSC survival. T16, Y27632 or untreated hiPSC were used in combination with various concentrations of the RhoA activator LPA. LPA treatment had no significant effect on the cell survival produced by any of the treatments, with untreated samples regardless of LPA concentrations, producing significantly lower cell survival (P=<0.001). Data shown as mean cell survival ± SEM, n=3.

4.2.4 T16 and Y27632 do not produce an additive effect

If T16 and Y27632 were targeting the same survival mechanism, it might be expected that using a sub-optimal concentration of either in combination would produce the optimal survival effect. However, this was not the case, when used together there was no additive effect on cell survival (figure 4.7). As expected, a 10μM treatment with Y27632 or 30μM T16 alone produced cell survival above 60%; a similar result was seen when these 2 doses were used in combination. Suboptimal concentrations (5μM Y27632 or 20μM T16) of either inhibitor resulted in survival figures greater than that of the untreated control, but still lower than those with the full treatment. Importantly however, the combination of low concentrations (2μM Y27632 or 10μM T16) of both, also resulted in survival of less than 30%. This apparent lack of an additive effect suggests that these survival compounds are not inhibitors of the same target kinase or pathway (ROCK for example). It is interesting to note that when used in excess (30μM) Y27632 treatment results in cytotoxicity. If T16 was targeting the same kinase as Y27632 it would be expected that full treatments of T16 and Y27632 (30μM and 10μM respectively) would also result in cytotoxicity, however this is not the case. This further suggests that T16 is not targeting the ROCK signalling pathway.

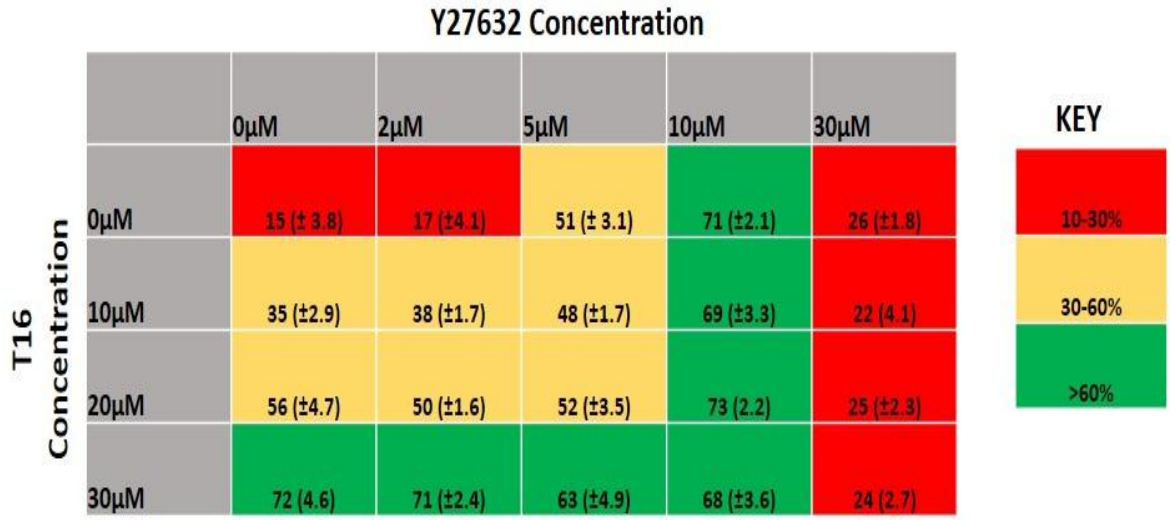


Figure 4.7-T16 and Y27632 used in combination. H1 (hESC) cells were treated in combination with various concentrations of T16 and Y27632 before being enzymatically passaged to determine if there was any additive between the survival compounds. Cells that had no survival compound, or with only 2μM Y27632 had cell survival of <30% as expected. Similarly, cells with 30μM T16 or 10μM Y27632 had >60% cell survival. Cells with sub-optimal concentrations of either compound had cell survival between 30-60%, even when used in combination. The high concentration of Y27632 impacted negatively on cell survival, which was <30%. Data shown as the mean survival, n=3.

4.2.5 T16 treatment does not result in the activation of Rac1 signalling

It has been reported that forced expression of GTP bound Rac1 could to protect hPSC from dissociation induced apoptosis (Ohgushi *et al*, 2010). Therefore, another potential mechanism through which T16 might act on the RhoA/ROCK/MLC axis is via the switch from high RhoA activity to high Rac1 activity. In order to analyse the levels of GTP bound Rac1 in response to dissociation, and to investigate if treatment with T16 alters this, an affinity bead based assay that specifically detected only GTP bound Rac1 was performed as described in section 2.4.5. The activation level of Rac1 was assayed 15mins, 30mins, 45mins, 2hrs and 8hrs post dissociation. Basal levels of GTP bound Rac1 was determined by lysing cells directly on the culture dish (not dissociated). Figure 4.8A shows representative immunoblots produced by western blotting. The data shows that levels of GTP bound Rac1 were unchanged between treatment types, with no significant differences observed at any of the time points analysed. All samples show some expression of GTP bound Rac1, with there being a general trend (although not significant) towards decreased activity in the final time point (figure 4.8B). This data suggests that treatment with T16 does not mediate its pro-survival effect by increasing the levels of GTP bound Rac1 and also shows that Y27632 treatment does not result in increased activity of Rac1 via antagonism of RhoA as reported in other cell types (Tang *et al*, 2012).

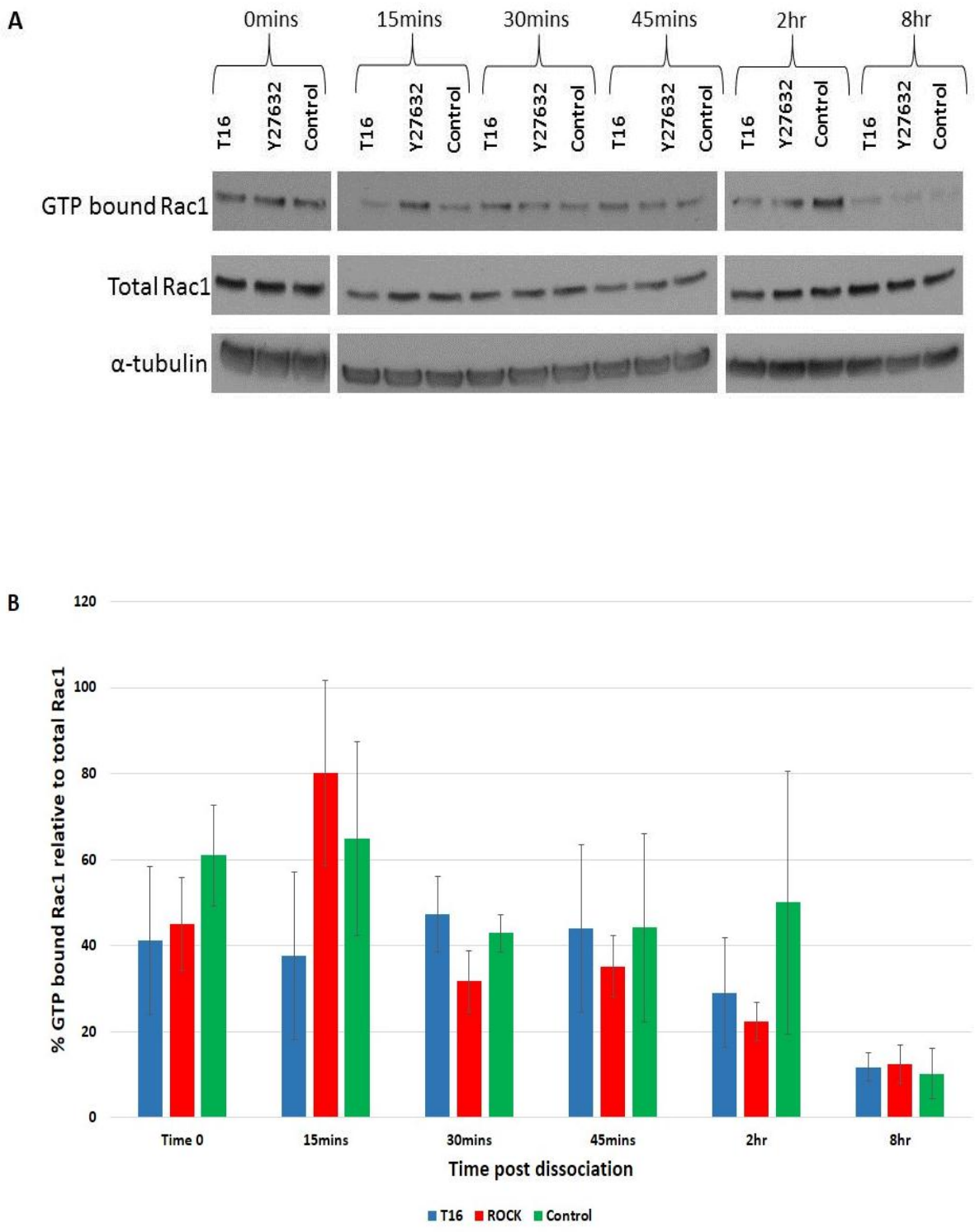


Figure 4.8- Levels of GTP bound Rac1 in response to dissociation. HiPSC (NMF-iPS6) were treated with T16, Y27632 or left untreated before being dissociated and harvested after 15mins, 30mins, 45mins, 2hrs and 8hrs. Samples were analysed for GTP bound Rac1 using Rac1 activation assay. Representative immunoblots produced show levels of GTP bound Rac1, total Rac1 and the loading control α-tubulin (A). Densitometry analysis of GTP bound Rac1 relative to total Rac1 expression (B**).** Data shown as mean values ± SEM, n=3.

4.2.6 T16 and Y27632 treated hPSC exhibit distinct morphologies

Upon replating, hPSC treated with the Rho kinase inhibitor Y27632 exhibited a very characteristic morphology. The cells appeared less well spread and had numerous spindle like projections when compared to untreated control cells. Notably, T16 treated cells did not exhibit this morphology, instead, they more closely resembled the untreated control cells. This can be seen in the phase contrast images below (figure 4.9). Untreated as well as T16 and Y27632 treated hPSC were enzymatically passaged as described previously and images taken 24hrs post passage.

The clearly identifiable differences in morphology between T16 and Y27632 treated cells is further evidence of discrete intracellular targets. The morphological difference is likely caused directly by ROCK inhibition in the Y27632 treated cells and downstream modification of cytoskeletal structure due to the established role of ROCK in regulation of the actin cytoskeleton, an activity that does not seem to be shared by T16.

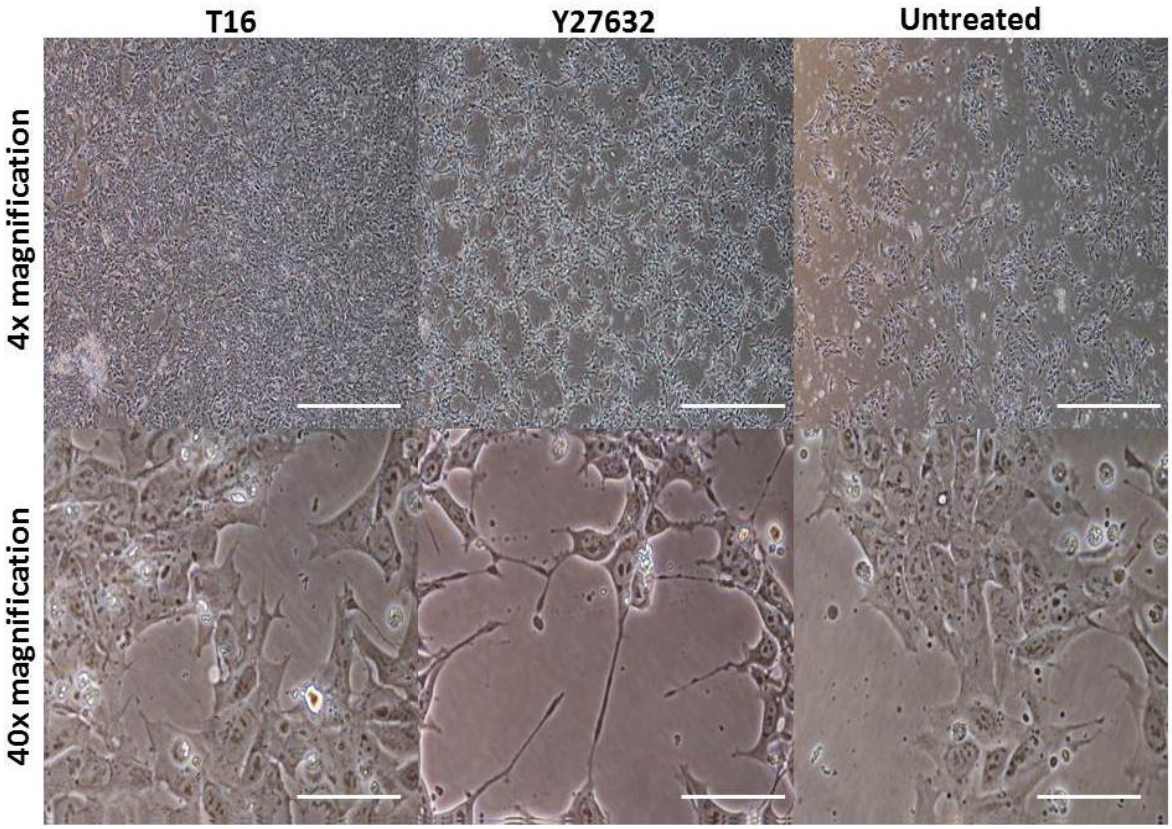


Figure 4.9- Distinct morphologies of T16 and Y27632 treated cells. HiPSC (NMF-iPS6) treated with T16, Y27632 or untreated control cells were passaged enzymatically and imaged under phase contrast microscopy 24hrs post passage. Scale bars represent 50µm.

4.2.7 Inhibition of the RhoA/ROCK/MLC axis is the cause of the spindle like phenotype

The prevention of the hyperphosphorylation of MLC has been reported as the cause of dissociation induced apoptosis of hPSC. As well as Y27632 mediated ROCK inhibition, the inhibitor blebbistatin has been shown to promote cell survival equivalent to that seen with Y27632 (Walker *et al*, 2010). Blebbistatin is a selective myosin II inhibitor which is also able to prevent the actinmyosin induced contractility reported to result in apoptosis of hPSC. To further investigate this characteristic morphology post dissociation in response to these established survival molecules, phase microscopy and immunocytochemistry were employed, with the FITC conjugated phallotoxin phalloidin used to image the F-actin within the cells.

Blebbistatin treated cells exhibit the same morphology as Y27632 treated cells (figure 4.10 c, h and m) and furthermore, inhibition of RhoA activation by C3 also results in this characteristic spikey morphology (figure 4.10 b, g and l). Only untreated (figure 4.10 e, j and o) and T16 treated cells (figure 4.10 a, f and k) exhibit a well spread morphology with well organised F-actin rich structures.

As hPSC treated with Y27632, Blebbistatin or C3 all exhibit this characteristic morphology, it is likely that the cause of this is the inhibition of downstream actinmyosin based contractility. Although the images shown in figure 4.10 were produced using hiPSC, these are representative of that observed with other hPSC lines such as H1, H9 and RC9.These findings are further evidence that T16 does not mediate its pro-survival effect via inhibition of the RhoA/ROCK/MLC axis, as treated cells do not exert the morphology associated with other inhibitors of this pathway.

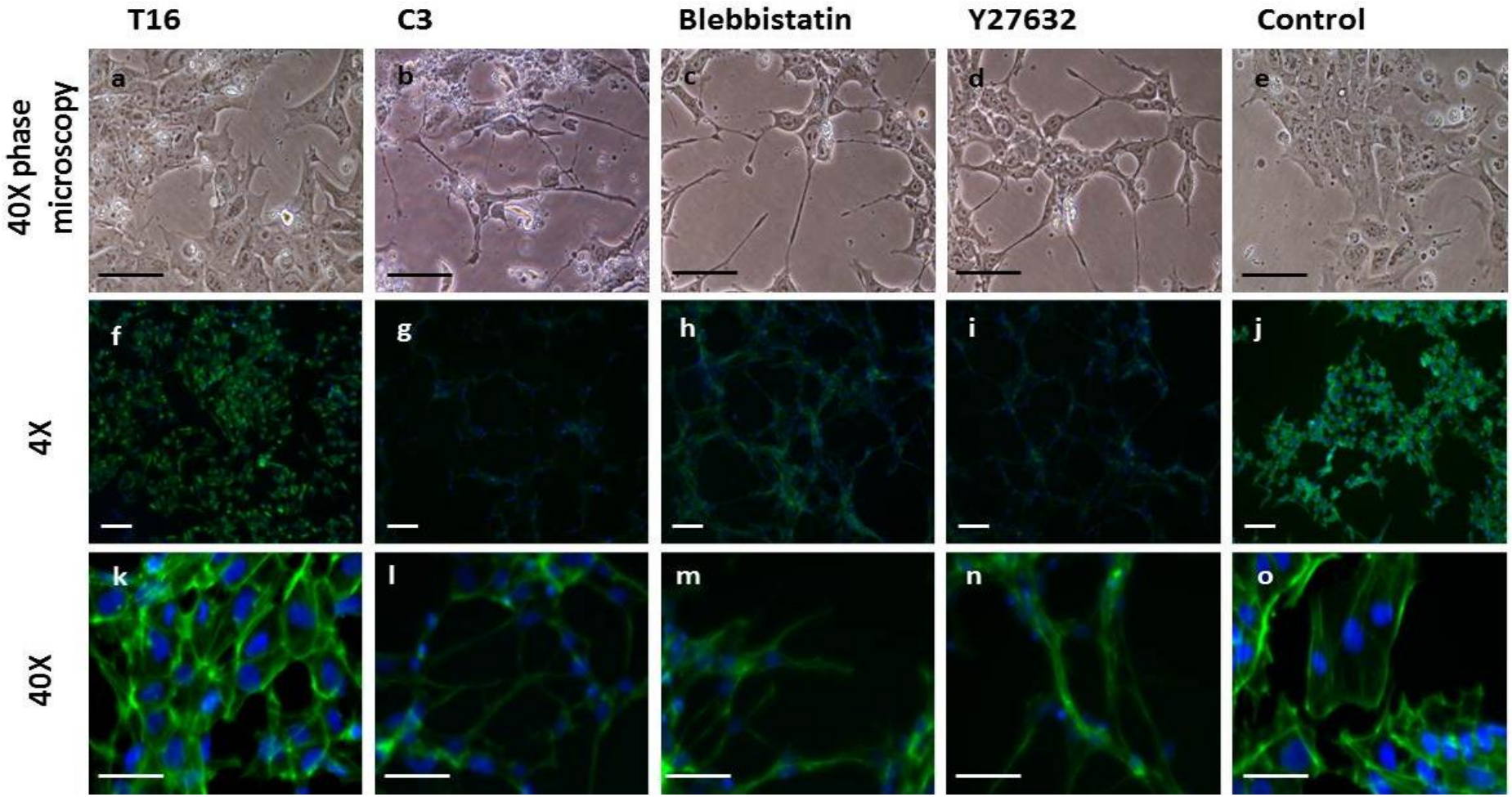


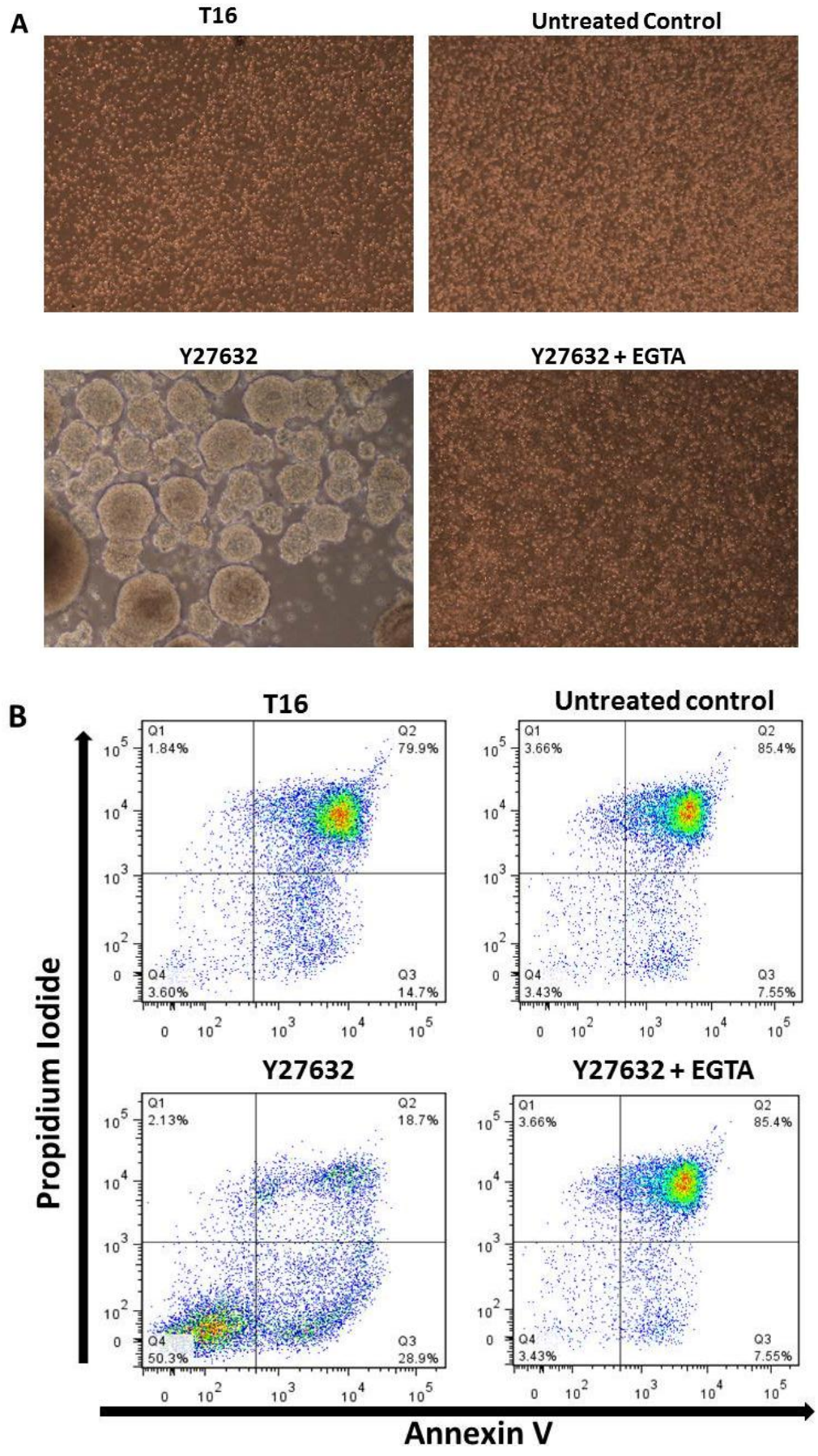
Figure 4.10- Morphological differences of T16 treated hPSC with alternative survival compounds. HiPSC (NMF-iPS6)) treated with T16 (a, f and k), C3 (b, g and l), Blebbistatin c, h and m), Y27632 (d, I and n) or untreated (e, j and o) were passaged enzymatically and were imaged by phase microscopy 24hr post passage before being fixed and stained with phalloidin (green) to target F-actin, and the nuclear stain DAPI (blue). The top row (a-e) shows 40X phase contrast microscopy images. The centre (f-j) and bottom (k-o) rows show immunofluorescence images at 4X and 40X magnifications. Scale bars represent 50µm on the top and bottom rows and 100µm in the centre row.

4.2.8 Survival of T16 treated cells is adhesion dependent.

During the original characterisation of Y27632, Watanabe *et* al (2007), described that in the absence of a suitable ECM or in non-adherent culture conditions, hPSC treated with Y27632 re-aggregate to form cell clusters in suspension. In order to test if this phenomena is observed after T16 treatment, hPSC were dissociated and replated in culture dishes which had no ECM coating, and could therefore not support attachment, and the formation of cell aggregates was assessed. To determine cell survival in response to this suspension culture, apoptosis assays were performed 24hr post dissociation as described in section 2.5.1. Phase microscopy images of hPSC 24hr post dissociation are shown in figure 4.11A. Only cells treated with Y27632 were able to form cell aggregates, with T16 treated and untreated cells remaining in single cell suspension. As observed by Krawetz *et al* (2009), the use of the calcium chelator EGTA (0.5mM) prevented Y27632 treated cells from forming cell aggregates (figure 4.11A).

Figure 4.11B shows representative flow cytometry plots showing that in the absence of a suitable ECM, T16 treatment is insufficient to prevent apoptosis, with 79.9% of cells being late apoptotic after 24hrs in suspension culture. Similarly, 85.4% of untreated cells are positive for both annexin V and propidium iodide and are therefore also late apoptotic. The formation of EB like clusters in response to Y27632 treatment prevented apoptosis in suspension culture with only 18.7% of cells being late apoptotic, however prevention of cell-cell adhesion by EGTA diminished the pro-survival effect of Y27632 treatment, with 85.4% of Y27632 + EGTA cells being late apoptotic (figure 4.12B).

The mean percentage of late apoptotic cells shows that there was a significant increase ($p=<0.001$) in survival of cells in suspension culture with Y27632 when compared T16, untreated or Y27632 + EGTA treated cell (figure 4.11C). This data shows a clear difference between drug treatments in response to suspension culture, which suggests a novel adhesion dependent mechanism of action for T16. These data are in agreement with that reported by others (Krawetz *et al*, 2009) also highlighted the importance of cell-matrix or cell-cell adhesion in the survival mechanism of Y27632, as if these adhesions are prevented, ROCK inhibition alone is insufficient to support hPSC survival despite the inhibition of pMLC.



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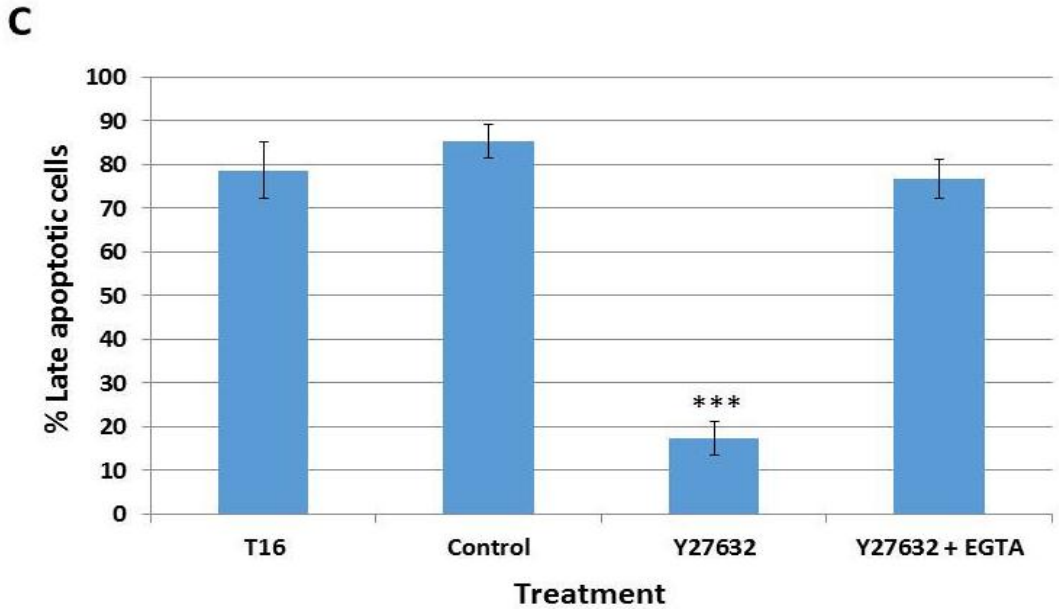

Figure 4.11- Response to suspension culture. Untreated, T16, Y27632 or Y27632 + EGTA treated hPSC (h1 hESC) were enzymatically passaged and replated in the absence of a suitable ECM. (A) Phase microscopy images (x4) show only hPSC treated with Y27632 alone were able to form cell aggregates resembling embryoid bodies. (B) Untreated, T16 and Y27632 + EGTA treated cells are >70% late apoptotic, staining positive for both annexin V and propidium iodide, whereas <20% of Y27632 treated cells are double positive. (C) Mean % of late apoptotic cells ± SEM (n=3) after the various treatment types. Treatment with Y27632 resulted in significantly reduced percentage of late apoptotic hPSC when compared to all other conditions (P=<0.001).

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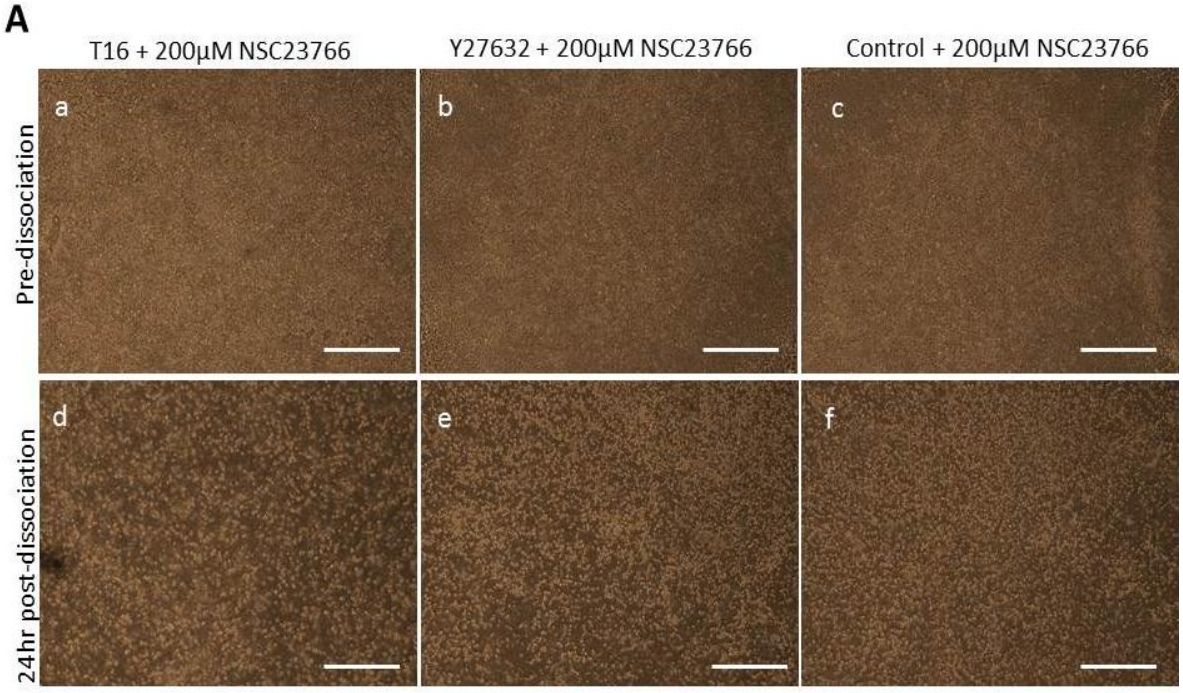
4.2.9 Rac1 activity is essential for adherence of hPSC post dissociation

As discussed earlier, the small GTPase Rac1 is a key component of the RhoA/ROCK/MLC axis and is often antagonistic to RhoA (Tang *et al,* 2012). Active GTP bound Rac1 has been shown to be a key component of cellular adhesion dynamics in a number of cell types, as well as being shown to have a protective effect on dissociated hPSC (Ohgushi *et al,* 2010), so to further investigate the role of Rac1 in hPSC attachment post dissociation, the Rac1 inhibitor NSC23766 was used. NSC23766 works by potent inhibition of Rac1 interaction with the Rac1 specific GEFs Tiam1 and Trio. Untreated, T16 and Y27632 treated cells (hiPSC) were enzymatically passaged in the presence or absence of various concentrations of NSC23766. Rac1 activity appears to be critical in the attachment and subsequent survival of hPSC, with concentrations of 50μM, 70μM, 100μM and 200μM NSC23766 all resulting in significantly reduction in the survival effect of T16 and Y27632 (P=<0.05 at 50μM, and P=<0.001 at 70μM,

100µM and 200µM). Additionally, NSC23766 when used at 70µM, 100µM and 200µM also significantly reduced the survival of control cells that had not received survival molecules (P=<0.05) (figure 4.12B). This observed decrease in survival was dose dependent and was unlikely to be a result of toxicity caused by NSC23766 as the inhibitor was incubated with the cells for 24hrs prior to dissociation without any adverse effects even at the highest concentration of 200µM, shown by healthy, normal monolayer of cells as imaged by phase microscopy (Figure 4.12A a, b and c).

Previous data has shown that in the absence of cell-ECM adhesion, Y27632 treated cells are dependent on cell-cell adhesion to promote survival (figure 4.11). Surprisingly, in the presence of NSC23766, Y27632 treated cells were prevented from forming both cell-ECM and cell-cell adhesions and remained in single cell suspension (figure 4.12 b and e).

These data confirm the critical importance of Rac1 mediated adhesion signalling in promoting the survival of hPSC, and also highlight an important role for Rac1 in forming cell-cell adhesions in Y27632 treated cells.



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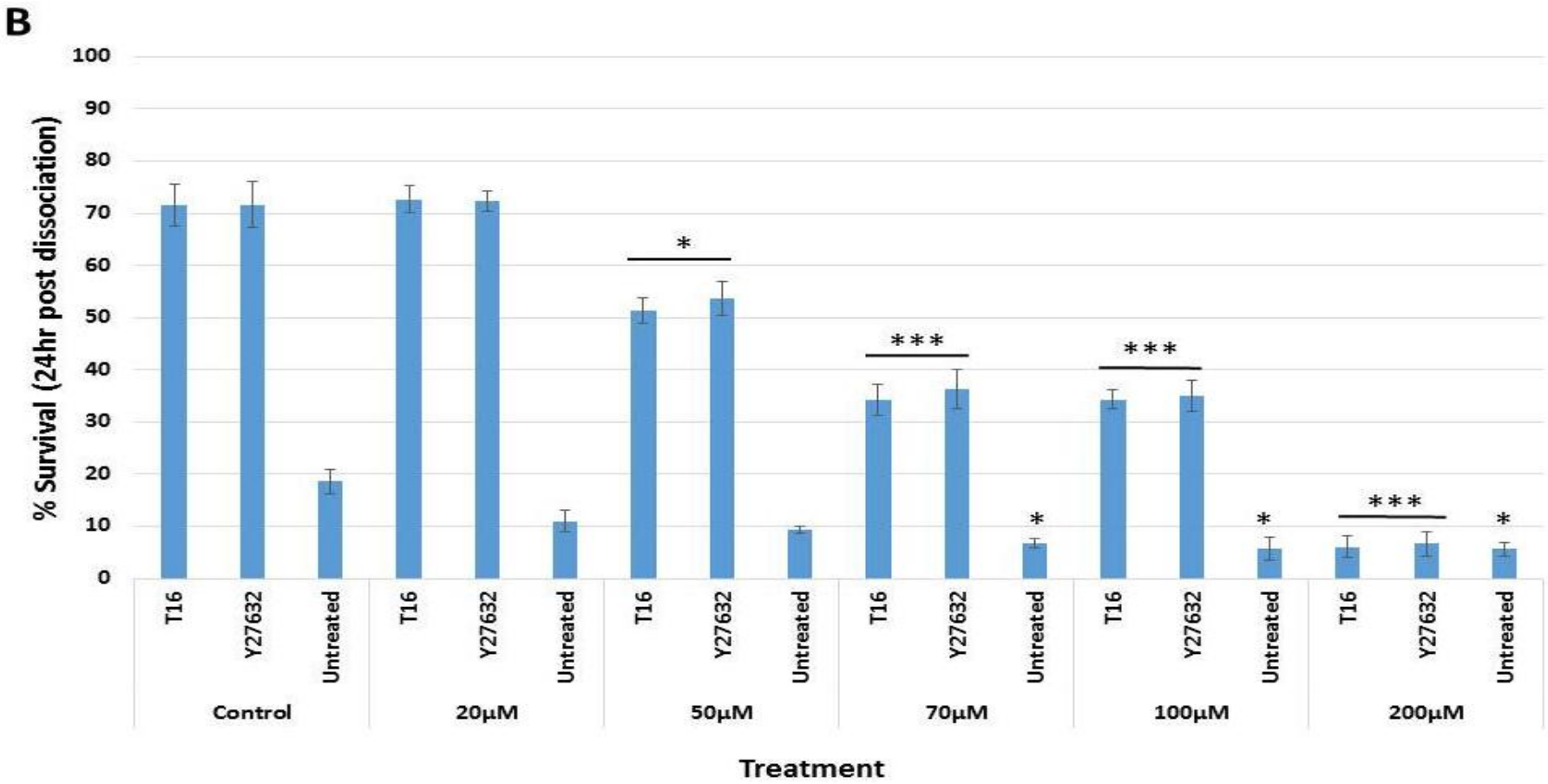


Figure 4.12- Active Rac1 is essential for hPSC attachment post passage. Phase microscopy images of hiPSC (NMF-iPS6) taken after 24hr incubation with 200µM Rac1 inhibitor NSC23766, showing no adverse effect on cell morphology or attachment (a,b and c). Phase microscopy images of T16, Y27632 and untreated hPSC in the presence of 200µM NSC23766, taken 24hr post dissociation (d, e and f) (A). Untreated cells and cells treated with T16 or Y27632 were enzymatically passaged in the presence or absence of various concentrations of the NSC23766 (B). A concentration of 50µM resulted in decrease in cell survival in T16 and Y27632 treated cells compared to the control cells with no NSC23766 (P=<0.05). Treatments of 70µM, 100µM and 200µM lead to decreased cell survival in T16 and Y27632 treated cells compared to the control cells with no NSC23766 (P=<0.001). Untreated control cells in the presence of NSC23766 resulted in decreased cell survival when compared to untreated control cells when used at a concentration of 70µM, 100µM and 200µM. Data shown as mean survival ±SEM , n=3.

4.3 Summary

Presented within this chapter are data that further characterise the pro-survival compound T16. An *in vitro* kinase assay showed that that T16 has very little effect on kinase activity, with RIPK2 the only kinase showing any degree of inhibition (72% inhibition at 30uM T16). The potential role of RIPK2 has been investigated using potent inhibitor of the downstream signalling complex NFκB, although endogenous RIPK2 protein expression could not be confirmed due to a paucity of reagents. Potential upstream or downstream modification of the RhoA signalling pathway by T16 was further investigated using western blots, RhoA and Rac1 activation assays and a combination of ICC and phase microscopy. Collectively these experiments have shown that T16 does not inhibit RhoA activation nor does it interfere with the activation state of Rac1 in response to dissociation. Furthermore T16 does not lead to a reduction in levels of phosphorylated MLC2, which is the previously published mode of action for Y27632, and the only mechanism by which hPSC avoid dissociation induced cell death that has been reported to date. Morphological studies showed distinct phenotypes in response to the different small molecules, with inhibition of actinmyosin induced contractility being strongly linked to the characteristic spikey morphology of Y27632 treated cells. Apoptosis assays demonstrated that the T16 mediated survival of disaggregated hPSC is dependent upon the reattachment of hPSC and may therefore be orchestrated by adhesion related proteins such as integrins. In addition, these data have highlighted an undescribed but critical role for Rac1 in mediating adhesion of both T16 and Y27632 treated hPSC to the ECM (and cell-cell in Y27632 treated cells). The data presented within this chapter provide strong evidence that T16 mediates is pro-survival effect via a novel RhoA/ROCK/MLC independent mechanism that is dependent upon cell-ECM adhesion.

5 T16 pathway analysis: T16 supports hPSC survival through an adhesion and Src-kinase dependent mechanism

5.1 General introduction

The body of data presented within chapters 3 and 4 shows that, despite promoting survival of disaggregated hPSC as efficiently as the commonly used ROCK inhibitor Y27632, T16 does not inhibit ROCK and has no effect on upstream regulators (RhoA and Rac1) or downstream mediators (MLC) of ROCK activity. This therefore suggests that T16 mediates its pro-survival effect in a RhoA/ROCK/MLC independent manner.

The survival effect of T16 has been shown to be dependent on signalling mediated by adhesion to the ECM, as in the absence of a suitable ECM, hPSC treated with T16 are still vulnerable to dissociation induced apoptosis. Experimentation was undertaken to further investigate the adhesion dependent survival mechanism mediated by T16.

The aims of these studies were to:

- Determine if treatment with T16 alters the expression of key mediators of cell adhesion either transcriptionally or at the protein level.

- Identify pathways of interest via kinase inhibition studies.

- Further investigate any pathways identified as potential mediators of the pro-survival effect of T16.

5.2 T16 treatment does not alter expression at the transcriptional level of a panel of adhesion related genes

Given that the pro-survival effect mediated by T16 has been shown to be dependent upon the reformation of cellular adhesions, expression of a panel of common adhesion related genes was examined via qRT-PCR. This gene list was chosen to include cell-cell and cell-matrix associated genes, as well as genes involved in scaffolding, focal adhesion complexes and downstream adhesion signalling. HPSC (hiPSC) were treated with T16, Y27632 or given no treatment before being lysed on the plate. RNA was harvested, cDNA produced as described in sections 2.3.1 to 2.3.3 and analysis performed using TLDA cards on the 7900 HT Fast Real-Time PCR System as describes in section 2.3.4. This method of quantification uses the ΔC_t value to calculate a $2^{\Delta Ct}$ x 1000 value which is plotted on graphs. Using this this method, a relatively high $2^{\Delta Ct}$ x 1000 of 400, would be indicative of a signal being detected within 1 cycle of the housekeeper, whereas a value of less than 0.1 would result from a signal being detected more than 15 cycles after the housekeeper. This method is particularly useful to give an indication of the absolute level of expression and relative quantification when an appropriate denominator is not available for the $2^{-\Delta\Delta CT}$ method, for example when a gene is not expressed in the control undifferentiated cells but is induced by treatment and therefore comparing expression would result in an infinite fold increase.

5.2.1 Transcriptional analysis of Integrin expression

Integrins are a very well characterised family of transmembrane proteins that play a central role in cell-matrix based adhesions. Integrins consist of both α and ß subunits and are major components of many signal transductions pathways, functioning both as ‘inside-out’ and ‘outside-in’ signalling proteins (Hu and Luo, 2012). Although there is a range of α-integrin subunits expressed in hPSC, there were no significant differences in expression in response to treatment type (figure 5.1A). ITGA6 (integrin α6) was the most highly expressed of the α-subunits, and although the difference did not reach significance, there was a trend towards increased expression in cells treated with T16. ITGA8 (integrin α8)

and ITGAL (integrin αL) were also included in the analysis, however there was no detectable expression of these subunits (up to 40 cycles). Analysis of the ß-integrin subunits analysed showed that there was no detectable expression of ß2 or ß3 integrins, and, as with the α-subunits, treatment with T16 or Y27632 did not induce any significant difference in the expression of ß-subunits (figure 5.1B). These data demonstrate that at the transcriptional level, treatment with T16 did not alter the expression of either α or ß integrin subunits.

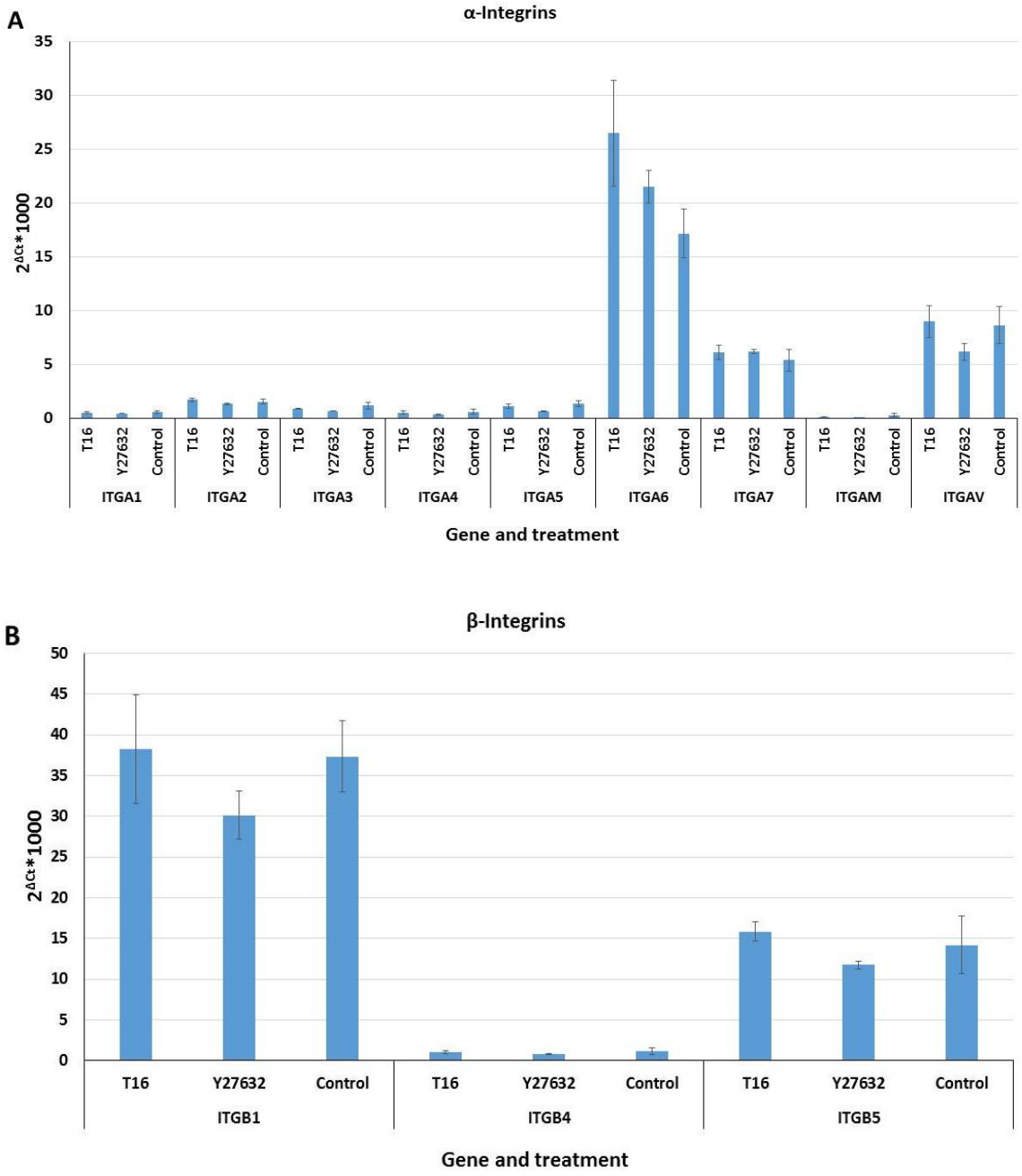


Figure 5.1- Transcriptional analysis of integrin expression. RNA was isolated from untreated, T16 or Y27632 treated hiPSC (NMF-iPS6) and analysed for integrin expression via qRT-PCR. (A) Shows there are a number of sub-units expressed, but that there were no significant differences in α-integrin expression between drug treatments and (B) shows no differences in β-integrin subunit expression. Data shown as mean $2^{\Delta Ct}$ x1000 ± SEM, n=3 in triplicate.

5.2.2 Transcriptional analysis of cadherin related proteins

The cadherin family of proteins are involved in homotypic cell-cell adhesions and have a number of intracellular binding partners associated with their function, including ß-catenin. Cadherins are known to play critical roles in signalling pathways mediated by GTPases such as Rac1 and RhoA (Wheelok and Johnson, 2003). In particular, CDH1 (E-cadherin) has been implicated in hPSC cell-cell adhesion (Ohgushi *et* al, 2010). PSC were found to have substantial expression of CDH1, CDH2 (N-cadherin) and CTNNB1 (ß-catenin) with CDH1 and CTNNB1 being more highly expressed than CDH2, however there were no significant differences in expression in response to the small molecule survival factors (figure 5.2).

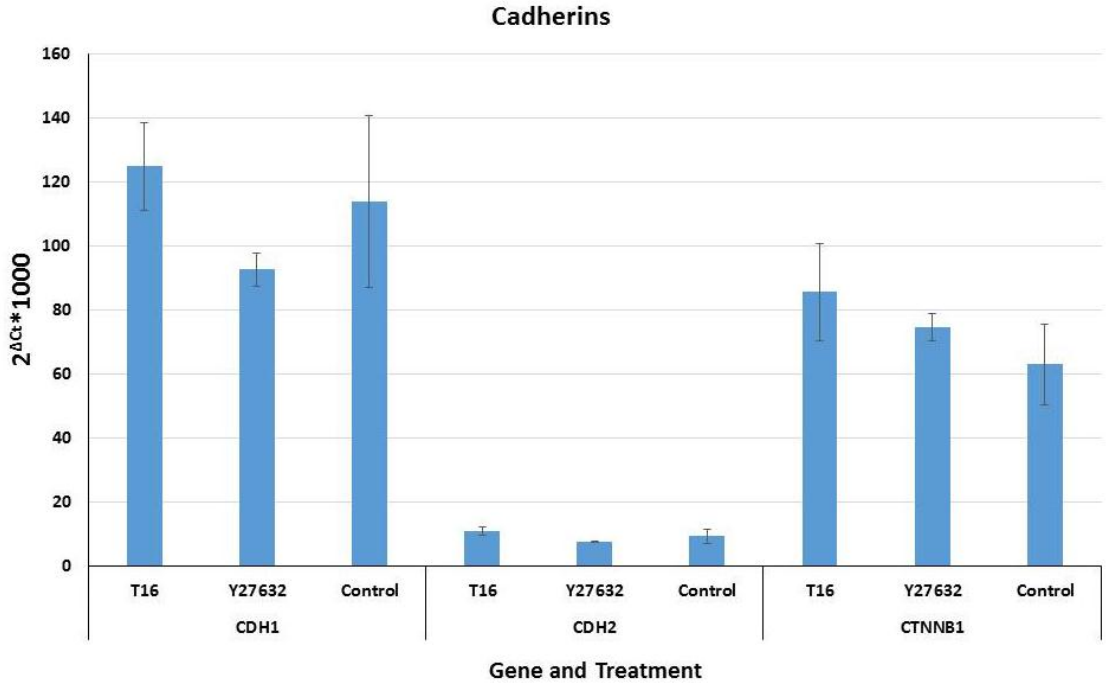


Figure 5.2- Transcriptional analysis of cadherin related proteins. RNA was isolated from untreated, T16 or Y27632 treated hiPSC (NMF-iPS6) and analysed for CDH1 (E-cadherin), CDH2 (N-cadherin) and CTNNB1 (β-catenin) expression via qRT-PCR. CDH1 was the most highly expressed gene, however there were no significant differences in CDH1, CDH2 or CTNNB1 expression over the three treatment types. Data shown as the mean $2^{\Delta Ct}$ x1000 ± SEM, n=3 in triplicate.

5.2.3 Transcriptional analysis of extracellular matrix related proteins

Expression of a range of the ECM related collagens and laminins was also analysed. Again, although there were detectable levels of many ECM related proteins (Figure 5.3), there were no significant differences between treatments in any of these genes. The most highly expressed ECM related genes were LAMA1, LAMB1 and LAMB2 (laminin α1, β1 and β2 subunits) (figure 5.3). This analysis also included the ECM proteins fibronectin and vitronectin, however there was no detectable expression of either of these matrix components.

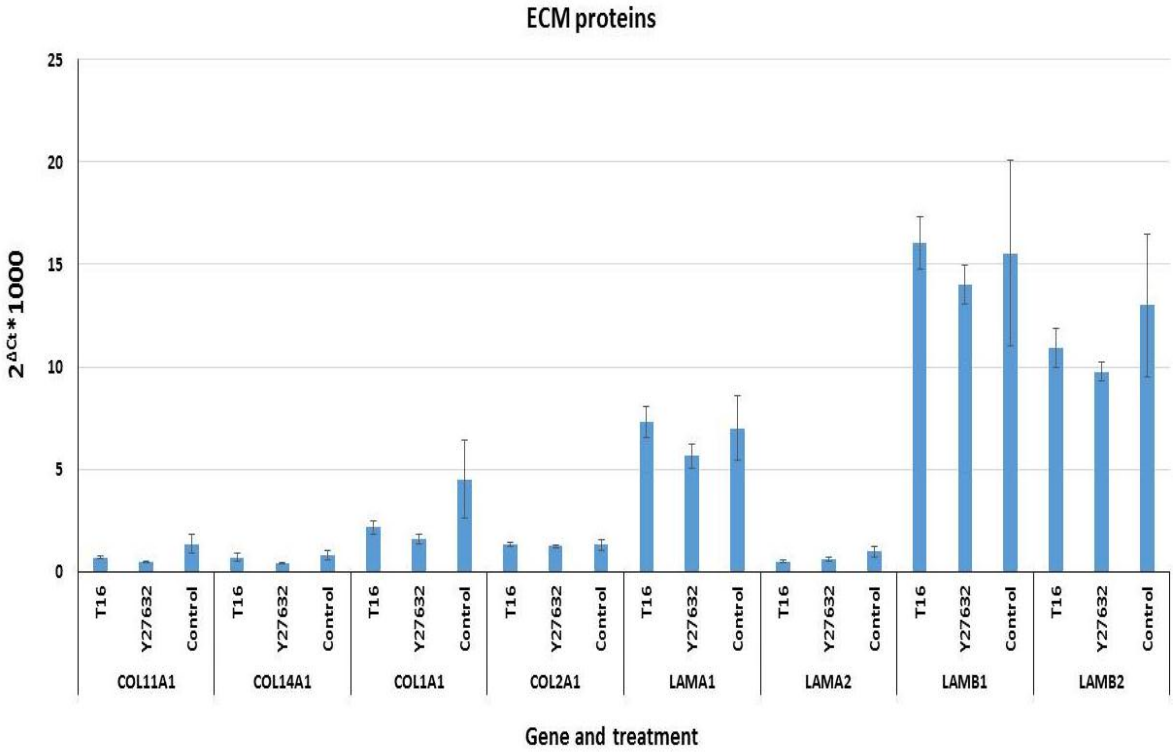


Figure 5.3- Transcriptional analysis of ECM proteins. RNA was isolated from untreated, T16 or Y27632 treated hiPSC (NMF-iPS6) and analysed for expression of genes associated with ECM proteins. Although there was expression in a number of these genes, there were no significant differences between drug treatments. Data shown as the mean $2^{\Delta Ct}$ x1000 ± SEM, n=3 in triplicate.

5.2.4 Transcriptional analysis of focal adhesion related proteins

As cell adhesion seems to play a critical role in survival, expression of key components of focal adhesion complexes such as PTK2 (FAK), VCL (vinculin), PXN (paxillin) and members of the Src-family of tyrosine kinases were also analysed. Figure 5.4A shows that each of the genes relating to focal adhesions were highly expressed in hPSC, however there were no significant differences after treatment with either pro-survival compound. There was also detectable expression of each of the Src-family kinases tested, Src, Fyn and Lyn (Figure 5.4B), with Fyn showing the highest level of expression of those family members that were analysed. As seen with the integrins and other adhesion molecules there were no significant difference in expression between treatment groups.

5.2.5 Connexin 43 and RACK1 were the most highly expressed adhesion related genes tested

From the analysis of this expanded panel of adhesion related genes, the gap junction protein GJA1 (connexion 43) and GNB2L1 (receptor of activated protein kinase C or RACK1) were the most readily detected, having a $2^{\Delta Ct}$ x1000 value of at least 3-fold greater than any other gene analysed. However, as with other genes analysed, there were no significant differences in the expression of these genes between treatments used (Figure 5.5).

The data presented in sections 5.2.1 through 5.2.5 show that at the transcriptional level, treatment with T16 or Y27632 does not significantly up-regulate or down-regulate the expression of any target in this extended panel of adhesion related genes. However, these data also provide more insight into the expression of adhesion related genes that are expressed by hPSC maintained in feeder-free culture conditions and highlight connexin 43 and RACK1 as extremely highly expressed genes in hPSC.

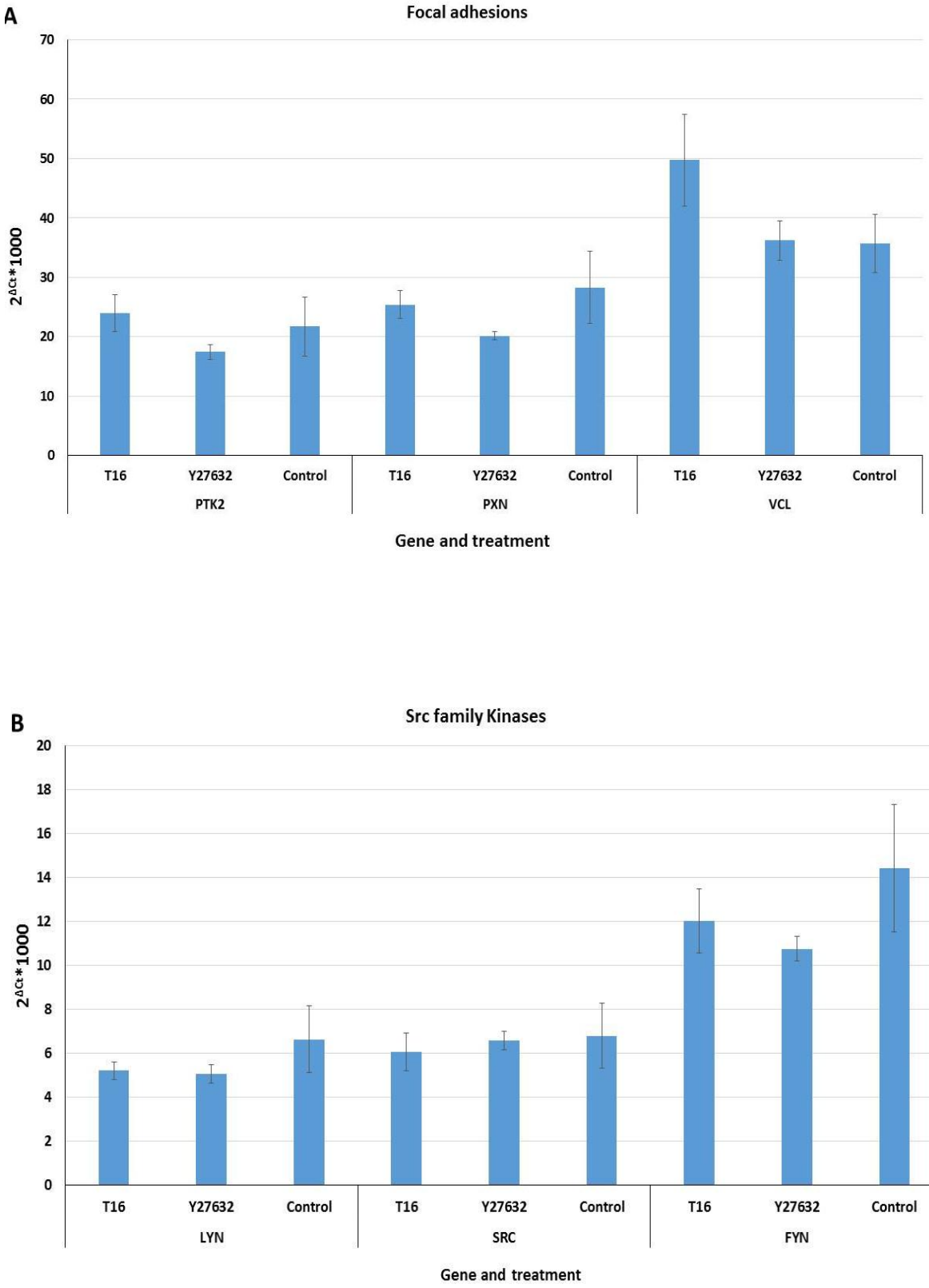


Figure 5.4-Transcriptional analysis of focal adhesion related proteins and Src-family kinases. RNA was isolated from untreated, T16 or Y27632 treated hiPSC and analysed for expression of genes associated with focal adhesions (A) or Src-family kinases (B). Although there was expression in each of these genes, there were no significant differences between drug treatments. Data shown as the mean $2^{\Delta Ct}$ x1000 ± SEM, n=3 in triplicate.

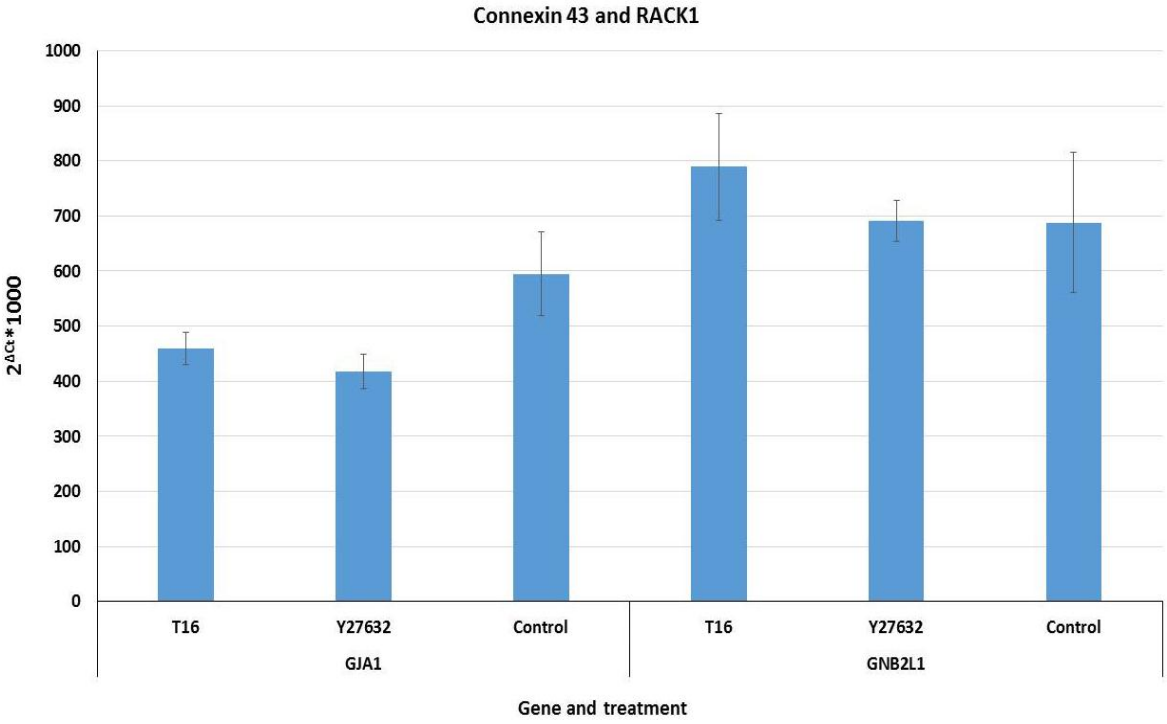


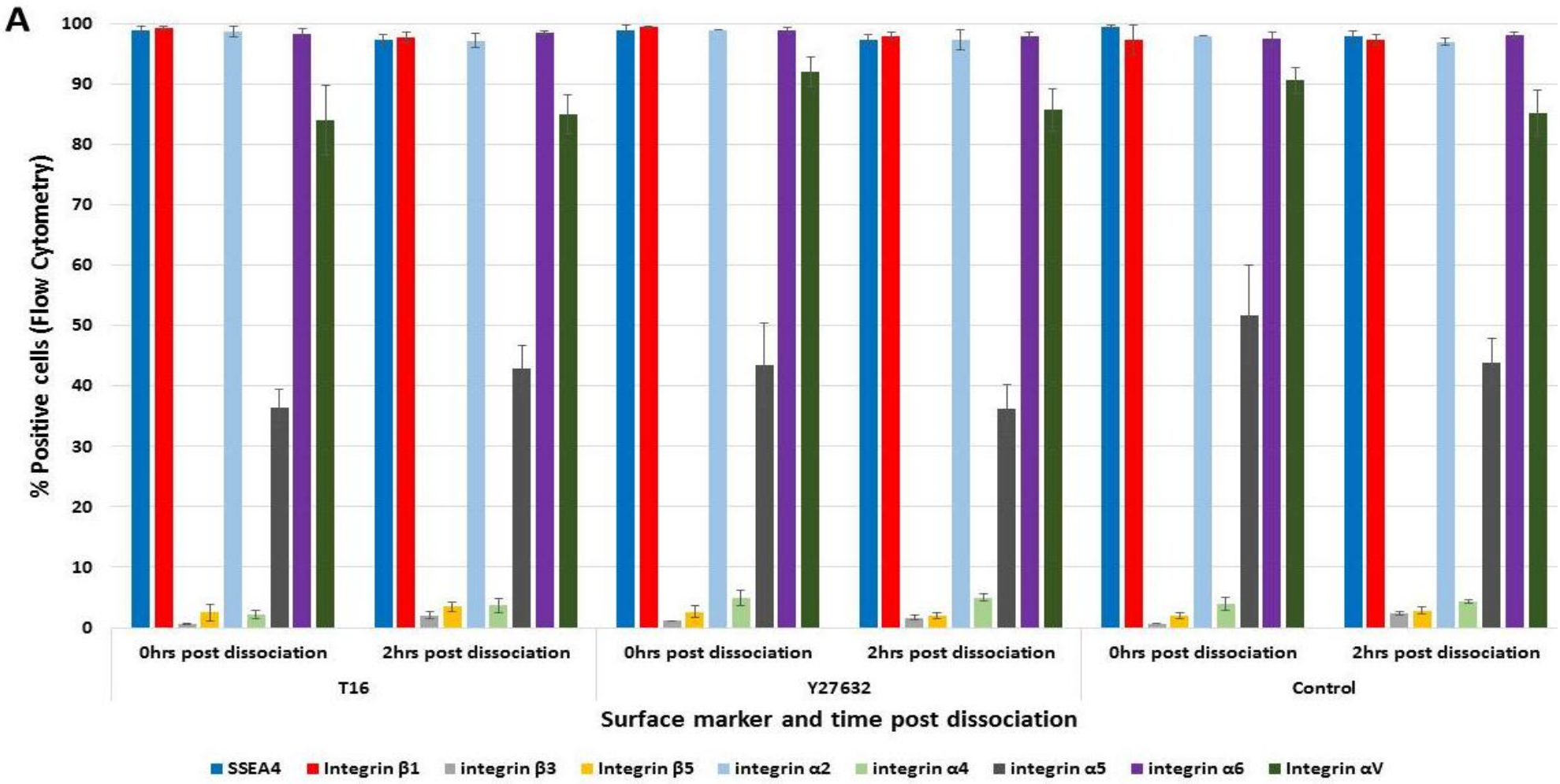
Figure 5.5- Expression of RACK1 and connexin 43. RNA was isolated from untreated, T16 or Y27632 treated hiPSC and analysed for expression of genes associated with cell adhesion. Of this panel of genes, connexion 43 (GJA1) and RACK1 (GNB2L1) had the highest expression, however there was no significant difference in expression between treatment types.

5.3 T16 treatment does not alter the expression of integrin proteins

Although T16 and Y27632 did not induce any change in the expression level of integrins at the transcriptional level, it was not clear whether there were any differences at the protein level. As it well established that the abundance of integrins at the cell surface can be regulated by recycling via endocytosis (as reviewed by Caswell *et al* (2009)), rather than by transcriptional control, it was particularly important to assess protein expression. It was possible that treatment with T16 or Y27632 prevented this internalisation and could thereby result in increased adhesion (and survival). Flow cytometry was employed to confirm the expression of integrin proteins at the cell surface and to determine if there were any differences in response to T16 treatment. SSEA4 was used as a control to confirm that the cells were pluripotent at the point of analysis. Untreated as well as T16 and Y27632 treated hPSC (hiPSC) were enzymatically dissociated and either analysed immediately, or left in suspension culture for 2hours then analysed. Greater than 95% of hPSC expressed the integrins ß1, α2 and α6 (figure 5.6A) regardless of treatment type. Additionally, >80% of hPSC had expression of integrin αV and 40-50% of cells expressed integrin α5. As seen in mRNA analyses, there was no significant difference in integrin expression between control and treated cells. Integrins ß2, ß5 and α4 were all expressed on fewer than 10% of cells in any condition.

As integrin internalisation and recycling may have altered the amount of protein expressed per cell rather than the % of cells that are positive, the mean fluorescent Index (MFI) for was also calculated for each sample. As illustrated in Figure 5.6B, using Integrin α6 as an example, no differences were observed in the MFI in any condition.

These data confirm that untreated hPSC do not internalise integrins (at least within the 2hr time frame used in this experiment) as there were no differences in the density of integrins detected at the cell surface, or in the % of cells expressing the integrin, after 2hrs in suspension culture (figure 5.6). These data therefore demonstrate that T16 does not mediate survival by up-regulating integrin expression or by preventing integrin internalisation upon dissociation.



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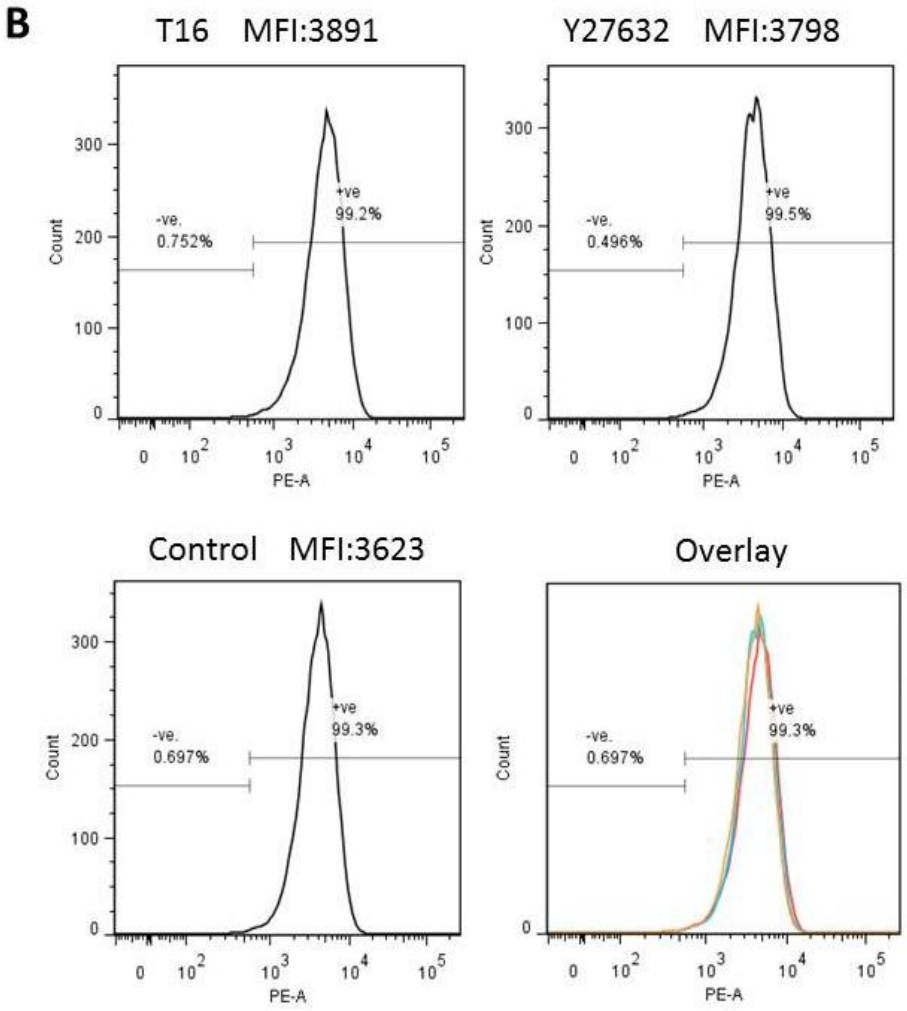


Figure 5.6- Changes in integrin expression between drug treatment and time post dissociation. HiPSC were treated with T16, Y27632 or left untreated before being dissociated and analysed by flow cytometry for expression of integrins. Cells were analysed immediately after dissociation and after a 2hr incubation under suspension culture conditions. SSEA4 was included to confirm the cells were pluripotent at the point of analysis. There were no significant differences in integrin expression between treatments or between time points (A). Histograms with MFI showing no difference in the amount of integrin α6 expressed in response to each treatment (B). Data shown as mean % of positive cells ±SEM, n=3.

5.3.1 Expression of integrins differ between hiPSC and hESC

Given the different methods by which hiPSC and hESC are obtained and initially cultured, it was possible that there would be a difference in the expression levels of integrins between these different cell types. To see if this was the case, hESC (H1) were compared to hiPSC for integrin expression as described above. Figure 5.7 shows that integrin expression between H1 and hiPSC was consistent for all integrins chains except integrin α5, with significantly fewer cells expressing integrin α5 in hiPSC ($p=<0.001$) despite the cells being maintained in similar feeder-free culture conditions for >10 passages before analysis.

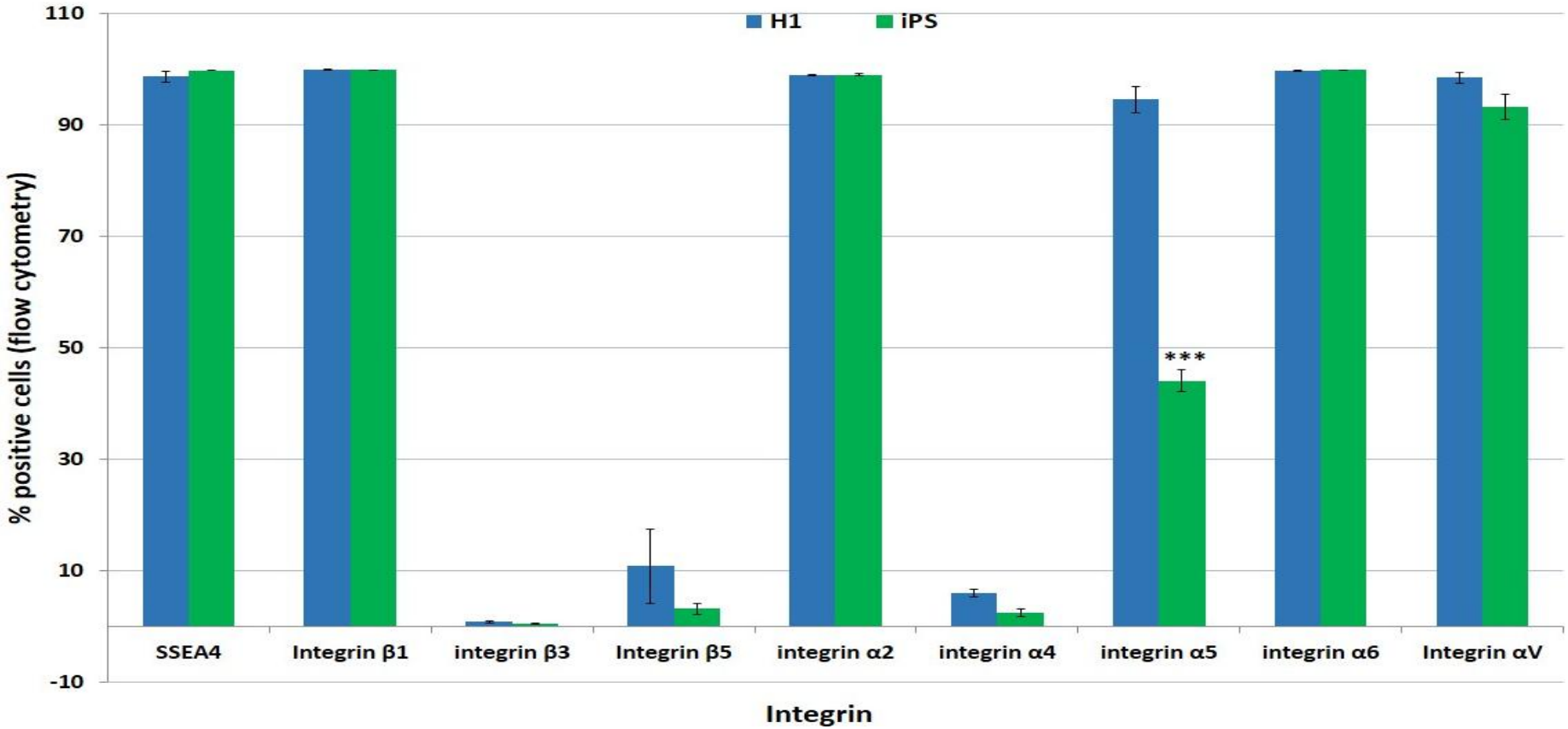


Figure 5.7- Integrin expression comparison between cell lines. H1 and hiPSC (NMF-iPS6) cells were analysed by flow cytometry to determine if there were differences in the expression of integrins. It can be seen that the expression of all integrins with the exception of integrin α5 have consistent expression between lines. Integrin α5 has significantly lower expression in hiPSC when compared to H1 cells (P=<0.001). SSEA4 was included to confirm the cells were pluripotent at the point of analysis. Data shown as the mean % of positive cells ± SEM, n=3.

5.4 Inhibition of potential pathways involved in survival highlights a role for Src kinase

Having shown that, despite its clear effect on cell-matrix adhesion, T16 does not appear to up regulate the expression of the adhesion related integrin proteins at either the mRNA or protein level, potential downstream signalling events were investigated. HPSC were pre-treated with T16 or Y27632 as previously described, and the survival factors were used in combination with a number of kinase inhibitors to test whether any of the inhibitors supported PSC survival alone or were able to abrogate the pro-survival effect of T16 or Y27632. Cells were then enzymatically passaged, with cell survival being assessed at 24hr post passage. The kinase inhibitors: LY364947 (TGF-ßR1), GF109203X (PKC α and ß1), API-2 (Akt), BIBX1382 (EGFR), PI-828 (Pi3K), ML-7 (MLCK) and PP2 (Src-family kinases) were used as described in section 2.2.1.4.

Treatment with LY364947, API-2, BIBX1382 or ML-7 had no effect on hPSC survival in the control cells, and did not alter the response of hPSC to either T16 or Y27632 treatment. Treatment of cells with the Pi3K inhibitor PI-828 resulted in a reduced capacity for T16 and Y27632 to support survival, with there being a significant decrease in hPSC survival in the presence of PI-828 compared to T16 or Y27632 alone (P=<0.001). In contrast, treatment with GF109203X had a significantpro-survival effect on untreated hPSC, resulting in an increase in cell survival from ~20% to ~45% (P=<0.001), however, this level of survival was still lower than that in reposnse to either T16 or Y27632 (figure 5.8).

However, the use of PP2 led to the greatest differential response. Inhibition of Src-family kinases by PP2 led to a significant decrease in cell survival of T16 treated cells (P=<0.001 versus all other T16 treatments) but had no effect on Y27632 mediated cell survival (figure 5.8) or control cells.

This specific abrogation of T16 mediated survival strongly supports a critical role for Src-family kinases in T16 mediated survival post dissociation and highlight another clear difference between T16 and Y27632. The findings also demonstrate that Pi3K and PKC signalling pathways have a role in the dissociation induced apoptosis of hPSC, with Pi3K activity supporting survival and PKC activity being pro-apoptotic.

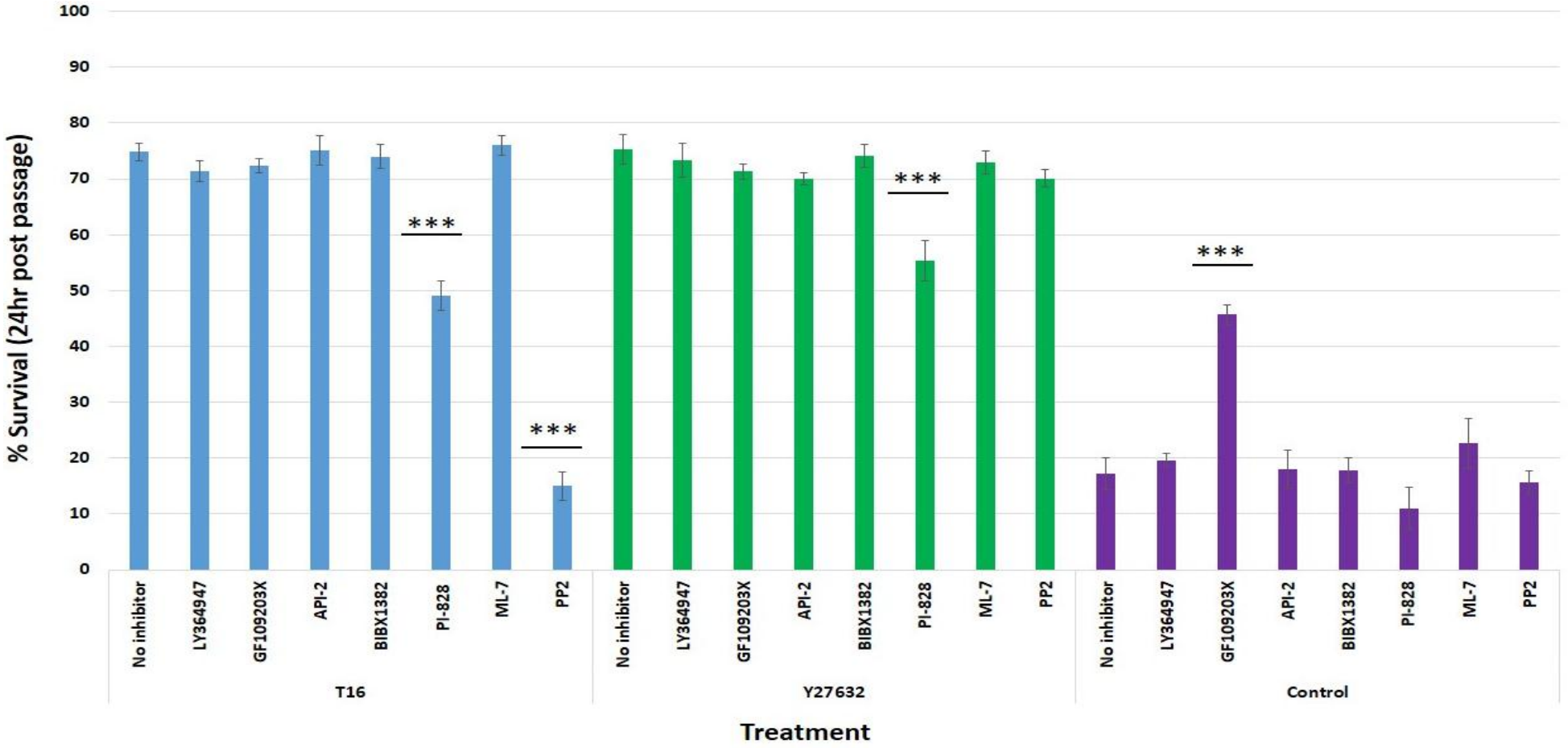


Figure 5.8- Effect on hPSC survival in response to kinase inhibition of various pathways. HiPSC (NMF-iPS6) were treated with T16 or Y27632 or left untreated and enzymatically passaged in the presence or absence of additional kinase inhibitors. Kinase inhibitors used were LY364947 (TGF-βR1), GF109203X (PKC α and β1), API-2 (Akt), BIBX1382 (EGFR), PI-828 (Pi3K), ML-7 (MLCK) and PP2 (Src-family kinases). PI-828 significantly decreased survival of Y27632 and T16 treated cells (P=<0.001) compared to all other Y27632 or T16 treatments. PP2 significantly decreased the survival of only T16 treated cells when compared to all other T16 treatments (P=<0.001). Inhibition of PKC through the use of GF109203X resulted in an increase in cell survival in untreated control cells compared to all other control treatments (P=<0.001). Data shown as the mean survival ± SEM, n=3.

5.4.1 Inhibiton of Src-kinases ablates the T16 survival effect in hESC and hiPSC

Given the striking effect of Src-kinases inhibition of T16 mediated survival in the hiPSC line used above, it was important to determine whether the inhibition of Src-kinases also resulted in a loss of the pro-survival effect of T16 in other hPSC lines. Therefore, the effect of Src-kinase inhibition on RC9, H1 and H9 hESC lines was tested by enzymatic passage with T16 or Y27632 in the absence or presence of PP2. Figure 5.9 shows that PP2 treatment has the same effect regardless of cell line used, ablating the pro-survival effect of T16 completely, and reducing cell survival to that of untreated control cells (P=<0.001). There was no significant decrease in Y27632 treated cells, confirming that this response was specific to T16 treatment.

5.4.2 The effect of Src inhibition is time dependent

Src-family kinases have well-known roles in adhesion and are major components of focal adhesions (Parsons *et al*, 2010). Given that the effect of T16 is dependent on adherence, experiments were undertaken to determine whether inhibition of Src-family kinases decreases survival by impacting adhesion related signalling. To do this, a time course experiment was performed during which PP2 was added to hiPSC at various time points. These included the standard 2hr pre-treatment, inclusion of PP2 directly after dissociation (0mins), and as addition of PP2 to cultures 30mins, 1hr, 2hr, 4hr and 8hr post dissociation. This experiment was performed with DMSO only (untreated) in addition to T16 and Y27632 treated cells.

As seen previously, addition of PP2 before or up to 2 hour post dissociation severely reduced T16-mediated survival, however addition of PP2 to cultures either 4hr or 8hr post dissociation resulted in a diminished effect of PP2, with hPSC survival of T16 treated cells being ~45% and ~60% respectively (figure 5.10). At the 8hr time point, the addition of PP2 had no significant effect on cell survival compared to T16 treatment alone. In the earlier time points (up to 4hrs post dissociation), survival was significantly lower than T16 treatment alone (P=<0.001), but there was a gradual increases that was dependent on the time post passage at which PP2 was added. During routine passage using survival

compounds, T16 treated hPSC begin to adhere to the ECM within the first 1-4 hours following dissociation, therefore, these results could suggest that Src-family kinase activity is required for this initial T16 mediated adhesion to the ECM, but that once cells have re-established contact with the ECM Src activity is dispensable.

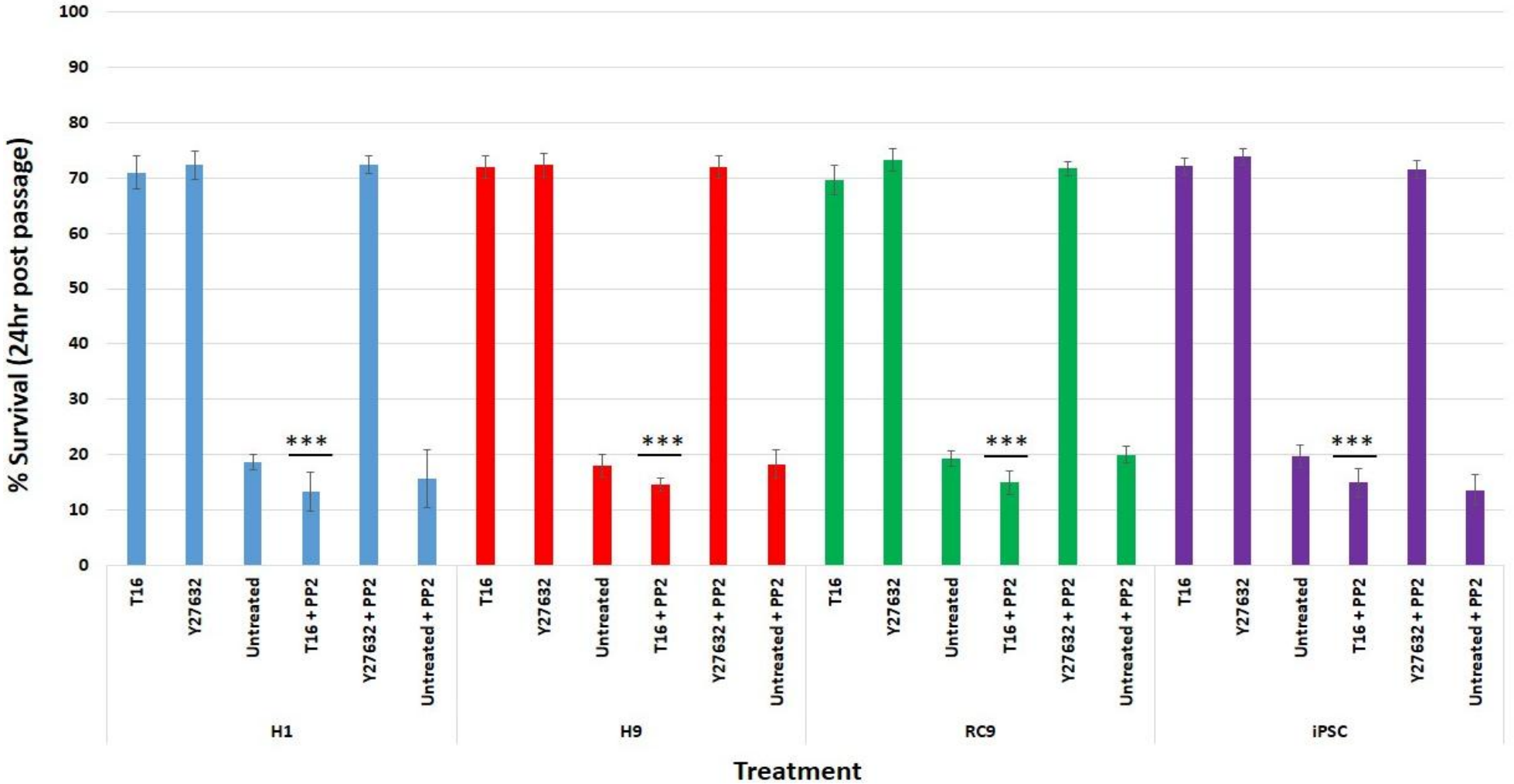


Figure 5.9- Effect of Src inhibition on hESC and hiPSC. H1, H9, RC9 and hiPSC were untreated or treated with T16 or Y27632 in the presence or absence of the Src-kinase family inhibitor PP2. In all cell types, PP2 significantly decreased the survival of T16 treated cells when compared to T16 alone, Y27632 alone and Y27632 with PP2 (P=<0.001). PP2 treatment had no effect on Y27632 treated cells. Data shown as the mean survival ± SEM, n=3.

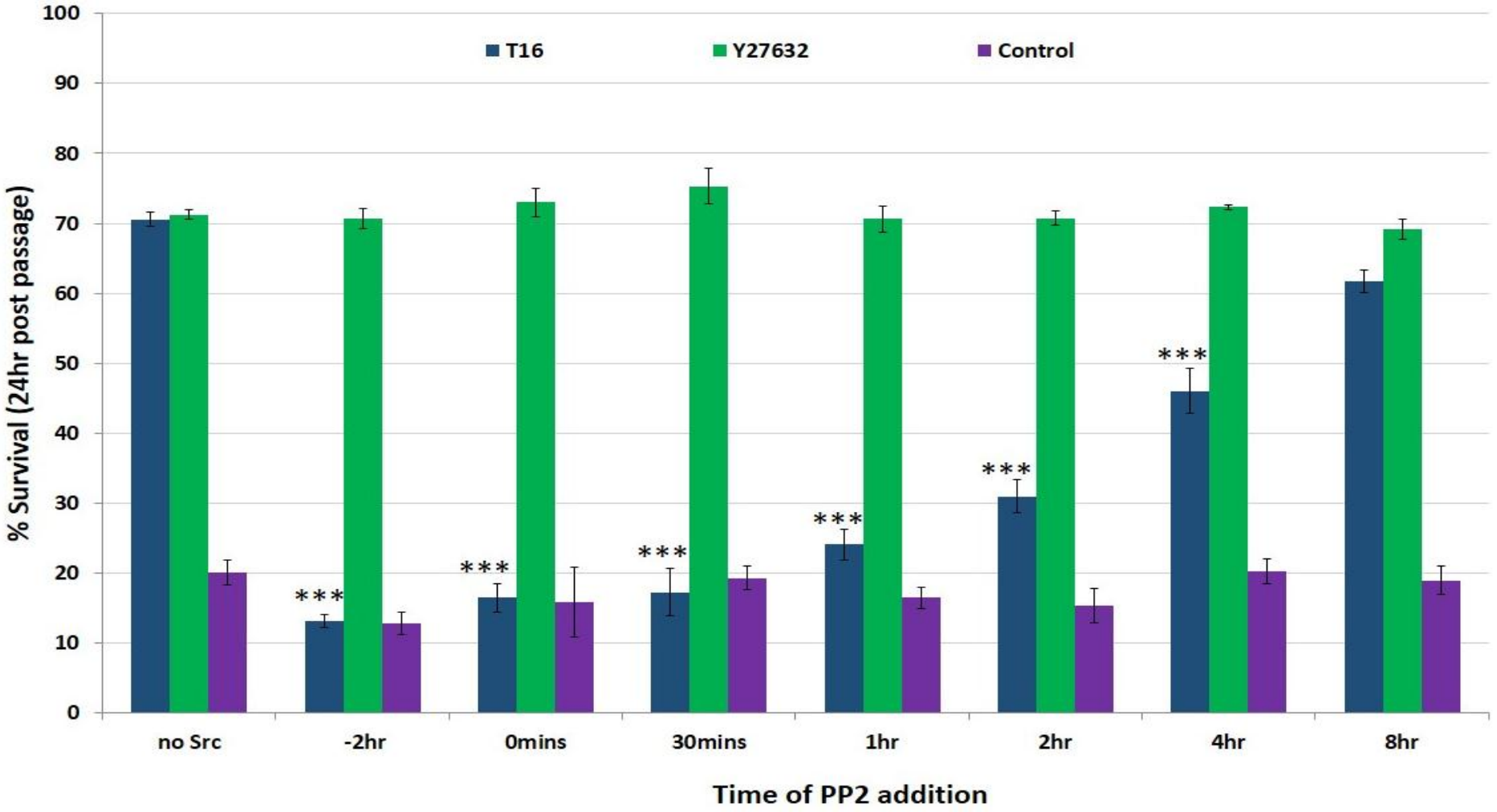


Figure 5.10- Time dependent response to Src inhibition. HiPSC (NMF-iPS6) were treated with T16, Y27632 or left untreated and passaged enzymatically. The Src-kinase inhibitor PP2 was added at various time points ranging from 2hr prior to passage to 8hrs post passage. T16 treated cells that had PP2 added between -2hrs and 4hrs post passage had significantly reduced cell survival compared to T16 treated cells without PP2 (P=<0.001). Data shown as the mean survival ± SEM, n=3.

5.4.3 T16 treatment does not alter the phosphorylation of src-kinases

The results of previous investigations demonstrate that Src-kinase activity is critical to the pro-survival effect of T16 in hPSC. In order to investigate if T16 treatment increased phosphorylation and therefore actvity of Src-kinases, T16, Y27632 or control hiPSC were enzymatically passaged and reseeded at a density of $7.5x10^5$ cells per well. Cells were harvested after 15mins, 30mins, 45mins, 1hr, 2hrs and 4hrs post dissociation and protein lysates produced as described in section 2.4.1. Gel electrophoresis and western blot analysis was performed as described in sections 2.4.3 and 2.4.4, and the resulting nitrocellulose membranes were probed with a pan-Src kinase antibody, a second antibody that recognisesphorphoylatyion at Y416 on multiple Src-kinases or the loading control α-tubulin antibody. Figure 5.11A shows example immunoblots for each protein target and figure5.11B shows mean densitometry data of pSrc relative to total Src-kinase. There was detectable expression of pSrc at each time point regardless of treatment type, however, there were no reproducible differences in expression between treatments or with increasing time post dissociation.

Further experiments were undertaken to confirm that treatment with the Src-kinase inhibitor PP2 (10μM) did result in the reduction Src-kinase phosphorylation that was expected and thus to validate the activity of this inhibitor. This was examined by performing western blot analysis on cells that were also treated with PP2 (figure 5.11C). The data produced confirms the capacity of PP2 to prevent phosphorylation of Src-kinase, with clear inhibition of pSrc but maintained detection of total Src-kinase in cells treated with PP2.

These data suggest that although Src-kinase activity is vital, T16 does not up-regulate the phosphorylation of this kinase and may therefore be influencing Src-kinase activity indirectly. Alternatively, it may suggest that T16 promotes survival downstream of Src-kinase, and that basal levels of phosphorylation are required for this activity. Although unusual this mode of action might be expected as it has previously been shown that T16 does not have significant kinase inhibitory activity (Chapter 4).

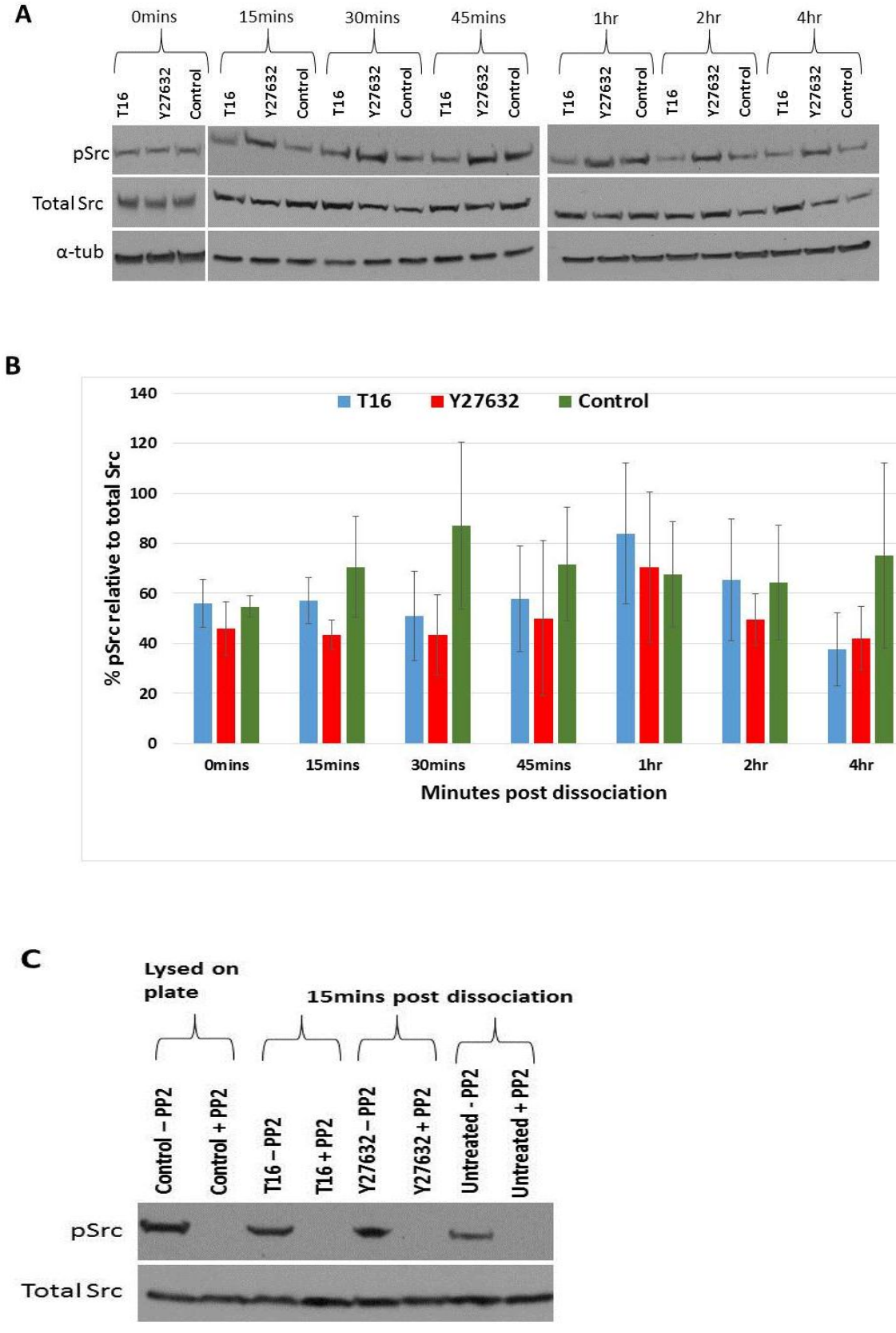


Figure 5.11- Western blot of Src-kinases activity. HiPSC (NMF-iPS6) were treated with T16, Y27632 or DMSO (untreated) before being enzymatically passaged. Cells were harvested and protein lysates made 0mins, 15mins, 30mins, 45mins, 1hr, 2hrs and 4hrs post dissociation. Western blots were performed and immunoblots produced. (A) Shows example immunoblots. (B) Shows densitometry analysis showing mean levels of pSrc relative to total Src (±SEM, n=3). Densitometry analysis showed there were no significant changes in the levels of active Src in response to treatment type. (C) Shows immunoblots confirming the inhibition of Src phosphorylation by PP2.

5.5 Proteome Profiler™ Antibody Array analysis of signalling pathways

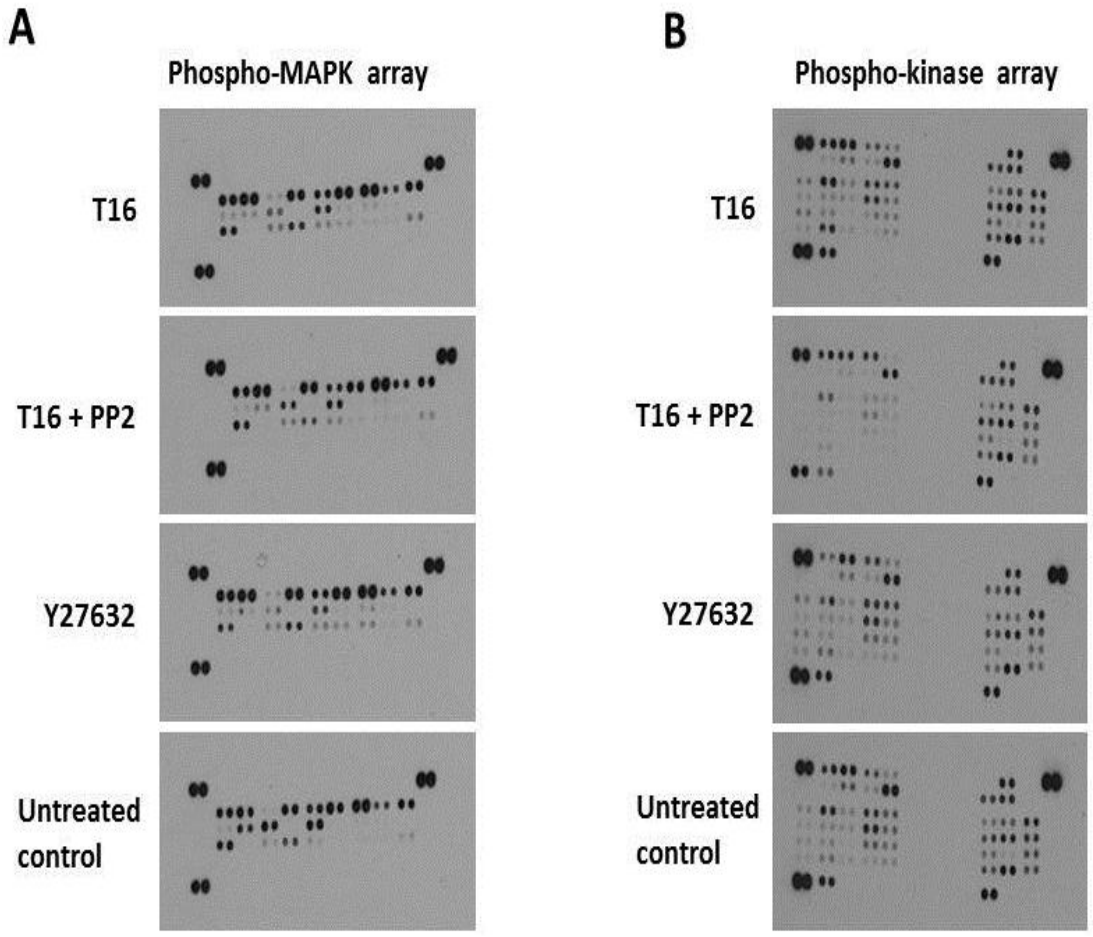
The preceding work shows that Src-kinase plays a critical role in T16 mediated hPSC survival, however, there were similar levels of phosphorylated Src present untreated hPSC that died post dissociation as in T16 and Y27632 treated cells which survived. It is therefore possible that the pro-survival effect of T16 is mediated either upstream or downstream of Src activation, and not via directly impacting the phosphorylation status of Src. The Src-family of kinases are involved in a wide range of cellular processes and are part of many signal transduction pathways. Given the large number of possible interactions, to further explore the pathways impacted by Src kinases and its inhibition by PP2 during hPSC dissociation, Proteome Profiler™ Antibody Arrays were used to investigate the activation state of 45 different phospho-kinases as well as a further 26 kinases involved in MAPK signalling. HPSC (hiPSC) were treated with T16, Y27632, T16 + PP2 or left untreated before being enzymatically passaged as described previously. Cells were harvested after a 2hr recovery period in fibronectin coated wells and then analysed using the Proteome Profiler kits as described in section 2.4.9. Proteome Profiler kits use nitrocellulose membranes that are spotted in duplicate with each protein of interest and the expression of each kinase can be subsequently determined by densitometry. The resulting immunoblots from the phospho-MAPK and phospho-kinase kits can be seen in figure 5.12A and B respectively. The immunoblots produced from the phospho-MAPK kit shows that the phosphorylation status of the majority of proteins was similar between the treatment groups. The only difference that was clearly identifiable by eye in pJnk1 and pJnk2, which appeared to have higher abundance in the untreated control cells and the T16 + PP2 cells. As with many MAPK proteins, Jnk proteins have been reported to play a role in apoptosis, however depending on cell type and family member, Jnk activity can be either pro or anti-apoptotic (Hochedlinger *et al*, 2002; Tafolla *et al*, 2005; Ries *et al*, 2008). Densitometry was performed on the nitrocellulose membranes and the mean pixel density plotted as shown in figure 5.12D. The densitometry unsurprisingly highlights those which were visible by eye, but also highlights other proteins with more modest changes such as a lower pAkt in control cells and an increase in pGSK3 in cells treated with T16 + PP2. These data provide

interesting insight, however as they are based on duplicate spots from an n of 1, care must be taken not to over interpret the data.

The same analysis was performed using the phospho-kinase array, and the resulting immunoblots can be seen in figure 5.12B. The most obvious difference on these immunoblots was that the T16 + PP2 sample had reduced phosphorylation in a large number of these kinases. There were no immediately obvious differences between the other conditions (T16, Y27632 and untreated control). When densitometry was performed on these immunoblots, the results, shown in figure 5.12C (i) and C (ii) confirmed that there was a decrease in many proteins in response to PP2. PP2 treatment not only reduced phosphorylation of Src-family kinases such as Src, Fyn and Lyn, but also reduced the phosphorylation of a number of other kinases such as FAK, PDGFR (platelet derived growth factor receptor), Erk1/2 and ß-catenin. This could be attributed to off-target inhibition by PP2, however it may also be a consequence of Src-kinase inhibition, given that many of these proteins are themselves targets of Src-family kinases. As with the MAPK array, it is important not to over analyse these data as they are from a single experiment, however there are a number of interesting differences highlighted they could be further investigated. For example, pChk-2 (checkpoint kinase 2) was higher in T16 treated cells than untreated or Y27632 treated cells, and expression of pChk-2 was substantially reduced in response to PP2 treatment. Chk-2 is involved in regulation of the cell cycle, and has a reported role in apoptosis (Janck *et al*, 2004), although largely thought to be pro-apoptotic, Chk-2 could have a novel role in hPSC behaviour post dissociation.

There is also increased detection of pRSK1/2/3 (ribosomal s6 kinase) in T16 treated cells compared to all other treatment types. RSK has been reported to prevent, or desensitise cells to, the release of cytochrome c and thereby reduces the apoptotic response (Kim *et al*, 2012). Between the 2 separate kits used, there was a certain degree of overlap between targets. The phospho-kinase kit utilises a pan pRSK antibody (RSK1/2/3) and shows T16 treated cells had the highest expression and that treatment with PP2 caused a decrease, however the MAPK kit used separate RSK1 and RSK2 antibodies which show no detectable expression in any treatment type. Taken together this would suggest

that RSK3 was therefore, the phospho-protein detected with the pan-RSK antibody in the phospho-kinase array and might have been a target for further investigation if time had permitted.



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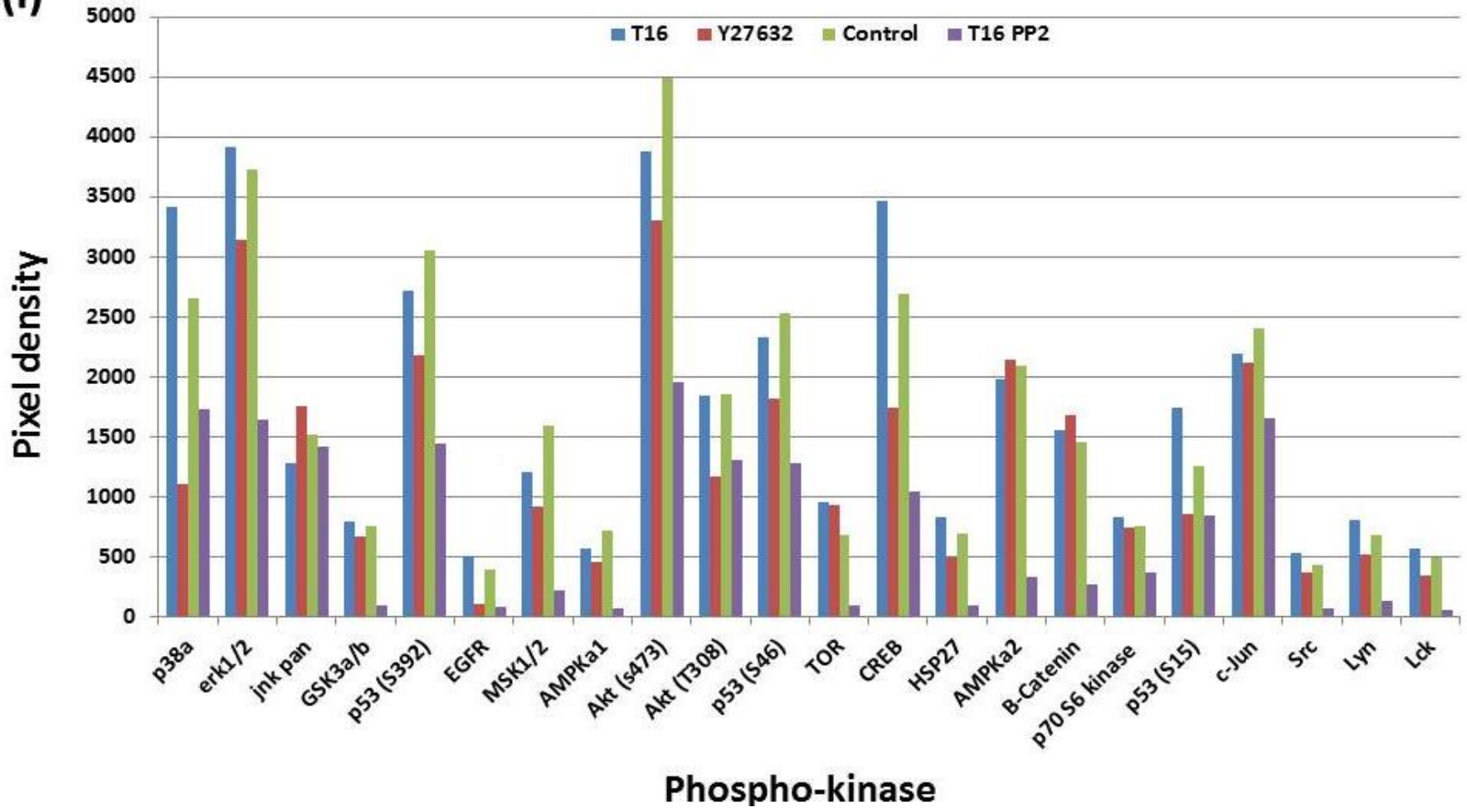
C(i)



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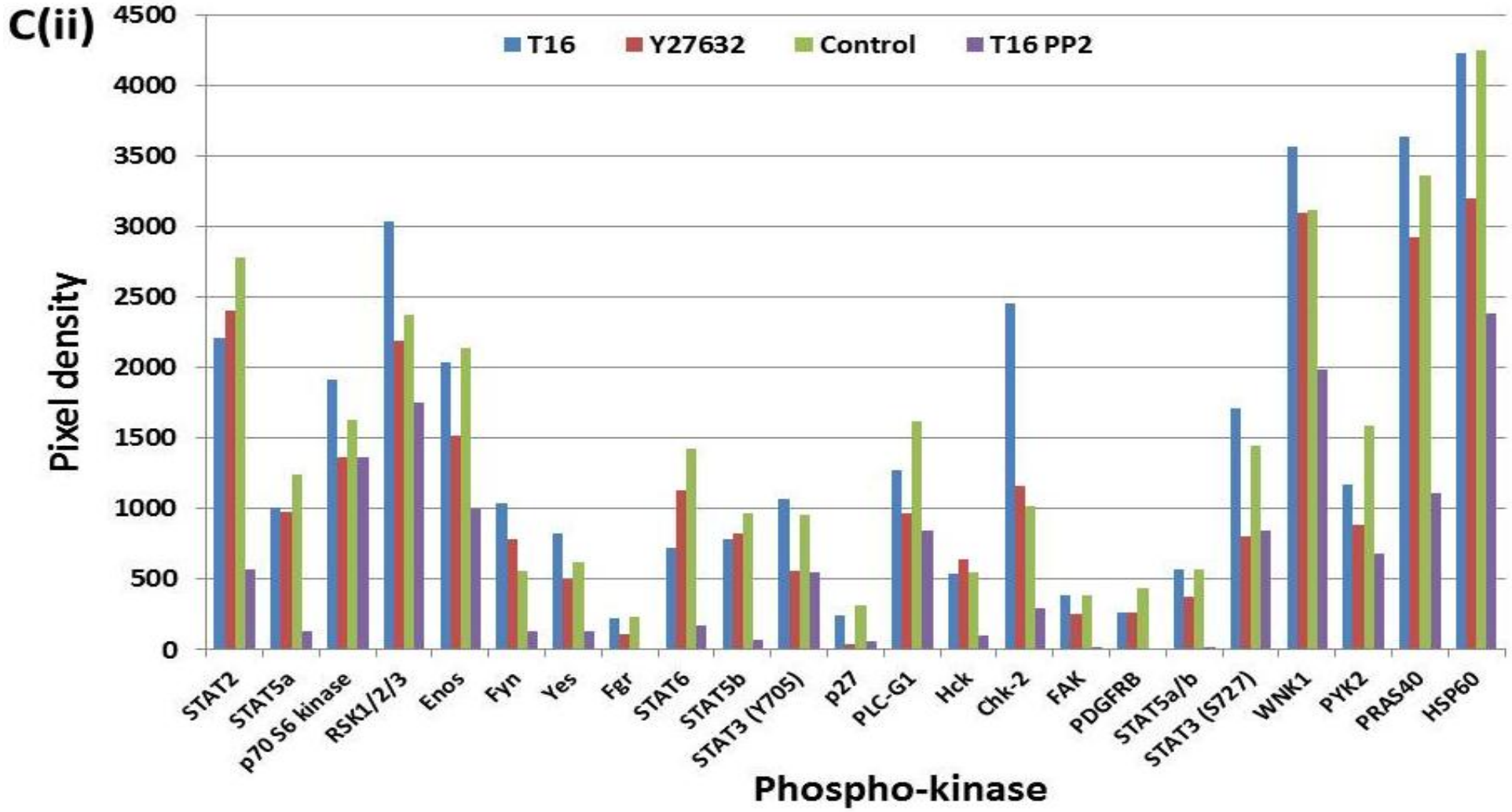

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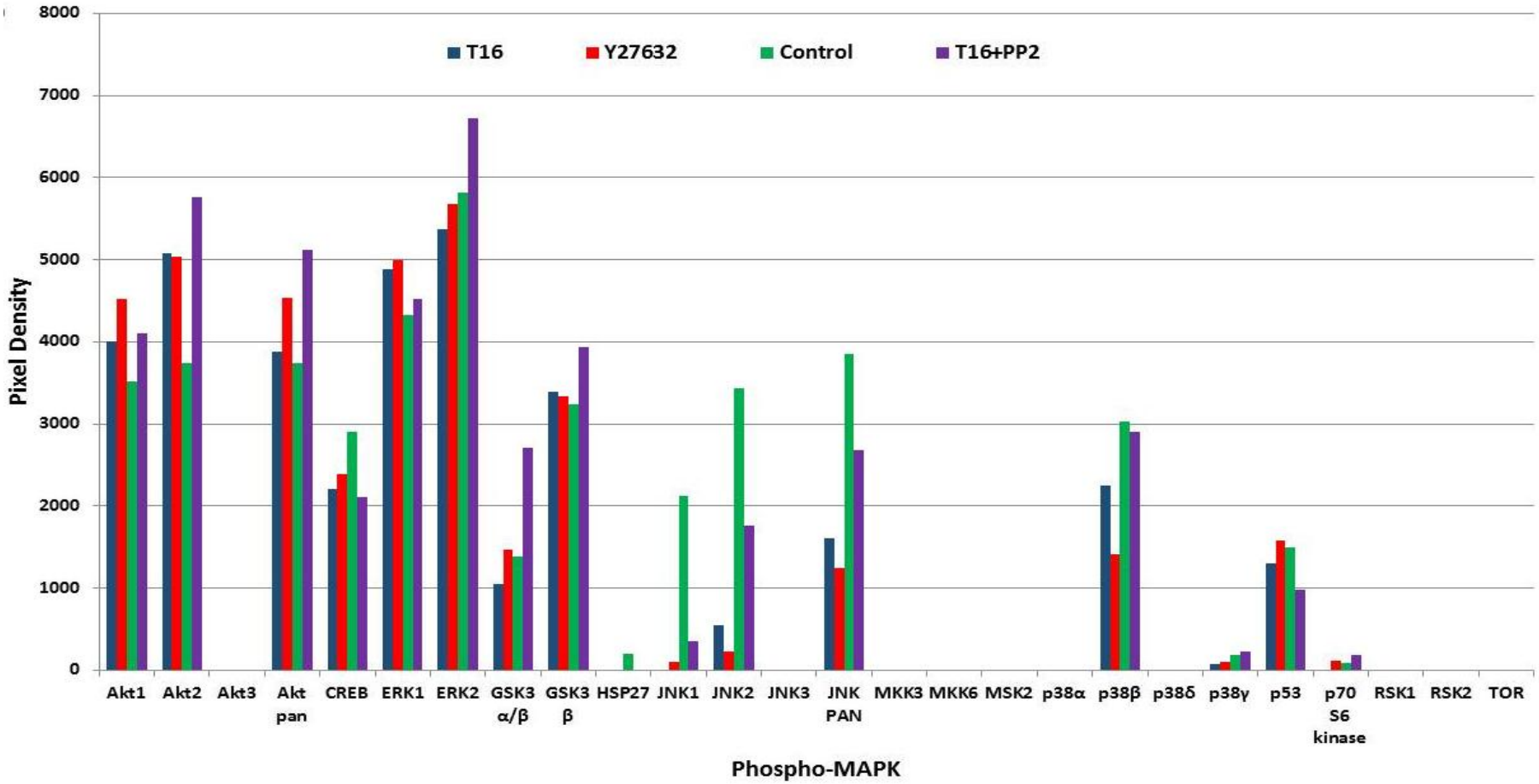


Figure 5.12- Proteome Profiler™ analysis. Untreated hiPSC (NMF-iPS6) as well as those treated with T16 ± PP2 and Y27632 were enzymatically passaged and cells harvested after a 2hr recovery period. Protein lysates were produced and analysed using Proteome Profiler kits. (A) and (B) show immunoblots produced by each of the kits. C(i) and (ii) show mean pixel density of targets from the phospho-kinase kit. (D) Shows mean pixel density of targets from the MAPK kit. Data shown as the mean pixel density from duplicate spots, n=1.

5.6 Potential role for RACK1 and Src interaction

5.6.1 HPSC express endogenous RACK1

Previous investigation using qRT-PCR had identified RACK1 as the most highly expressed adhesion-associated gene in hPSC (figure 5.5). RACK1 has been reported to have the capacity to bind members of the Src-kinase family and consequently to be able to mediate the activation of signalling pathways downstream of Src-kinase including those involved in adhesion and apoptosis, for example Pi3K/Akt signalling (Liu, *et* al, 2011; Mamidipudi *et al*, 2006).

Experiments were undertaken to investigate whether there is any direct interaction between the RACK1 and Src proteins in hPSC. Firstly, in order to confirm if hPSC express RACK1 protein, hPSC were grown to confluence before being lysed as described in section 2.4.1. Western blot analysis of RACK1 in the hESC lines H1 and H9, as well as hiPSC, was performed as described in section 2.4.4. As shown in figure 5.13, all of the hPSC lines expressed significant amounts of RACK1.

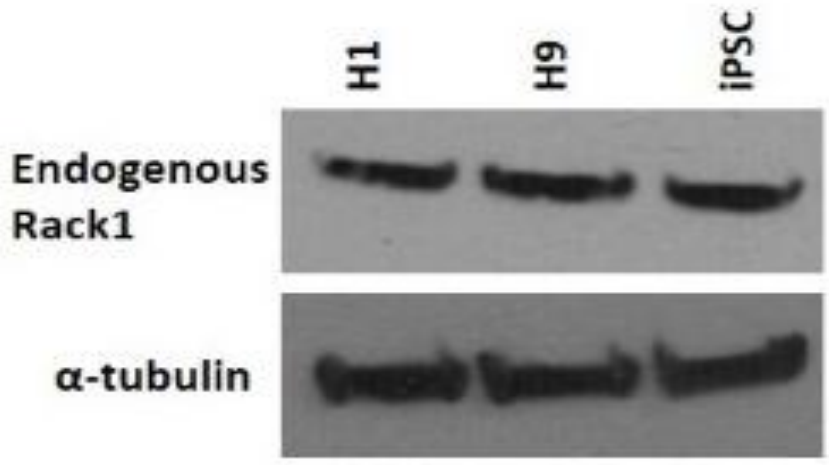


Figure 5.13- HPSC express endogenous RACK1. H1 (hESC), H9 (hESC) and hiPSC (NMF-iPS6) were lysed and protein lysates produced. SDS PAGE and western blot analysis were performed and the resulting nitrocellulose membranes probed for RACK1 and α-tubulin. RACK1 was present in each cell line tested (n=1).

5.6.2 T16 treatment alters Src binding capacity to RACK1

To assess if Src-family kinases had the capacity to bind RACK1 in hPSC, a GST RACK1 fusion protein was produced and purified as described in section 2.4.8. T16, Y27632 and untreated control cells were used to produce protein lysates using non-denaturing lysis buffer. An aliquot of each was taken as a total lysate control before the remaining lysate was incubated with the GST RACK1 fusion protein for 1hr at 37°C in the presence of ATP and kinase buffer. This was done to ensure the phosphorylation of the RACK1 fusion protein, which is reported to be necessary for association with Src-family members, and to facilitate binding of any free cytoplasmic Src-family kinases (Chang *et al*, 2002). Glutathione beads were then used to separate the GST RACK1 and any binding partners from the lysate before denaturing SDS-PAGE and western blots were performed on both the pull-down samples and total lysates. The resulting immunoblots were probed with anti-Src (total, pan-Src), anti-RACK1 and anti-α-tubulin antibodies, with representative immunoblots shown in figure 5.14A. These immunoblots confirm that the GST RACK1 fusion protein successfully binds to endogenous Src proteins in hPSC, although with varying levels. Densitometry analysis of pulled down Src proteins is shown in figure 5.14B and demonstrates that the Y27632 treated and the untreated control cells GST-RACK1 pulled down significantly more Src protein compared to the T16 treated cells ($P=<0.01$ and $P=<0.001$ respectively). This difference is not a result of differing levels of input Src-family kinase as there was no significant difference in expression between the three treatment types (figure 5.14C), thus T16, but not Y27632, does appear to modulate the interaction of RACK1 and Src-family kinases in dissociated hPSC.

Although the introduction of exogenous RACK1 makes this system somewhat artificial, these data highlight an interesting difference between the treatment types, and suggest that T16 treatment reduces the capacity of one or more Src-kinases to bind to RACK1. This is likely a result of Src proteins already being bound to other intracellular targets such as integrins, FAK or other survival related proteins.

It was also possible that reduced level of Src-family kinases was pulled down by GST-RACK1 in T16 treated hPSC because Src family members were already bound

to endogenous RACK1 and therefore not available to the fusion protein. The association of endogenous proteins was therefore further investigated.

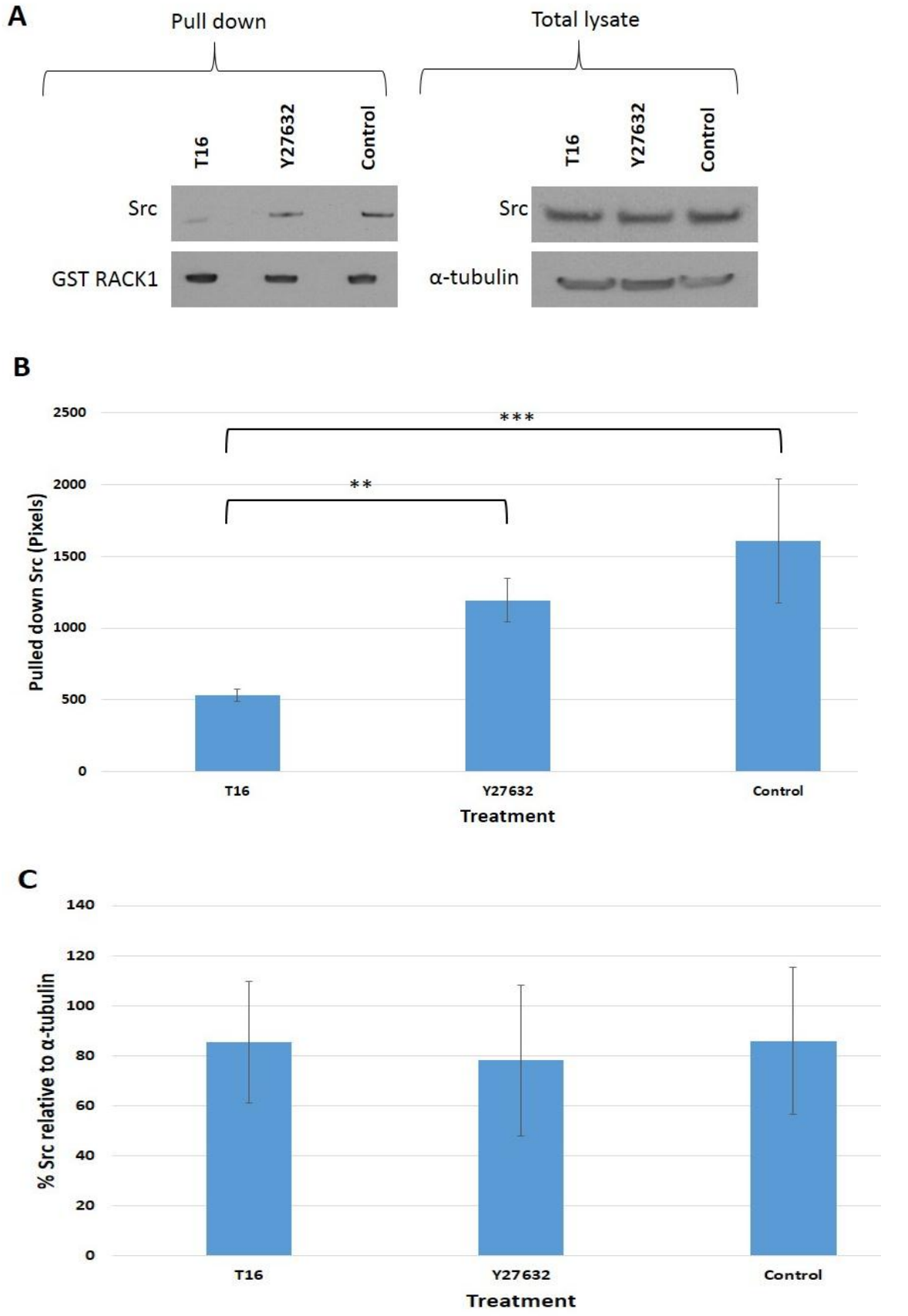


Figure 5.14- Pull down of Src-kinases using GST-RACK1 fusion protein. A GST fusion protein was incubated with lysates produced with T16, Y27632 or untreated cells (NMF-iPS6) for 1hr in the presence of ATP and kinase buffer before being analysed by western blotting. (A) Representative immunoblots produced. (B) Densitometry analysis showing significantly less Src proteins pulled down in T16 treated cells when compared to Y27632 (P=<0.01) or untreated (P=<0.001) samples. (C) Densitometry analysis of total lysates showing equal loading of total Src protein.

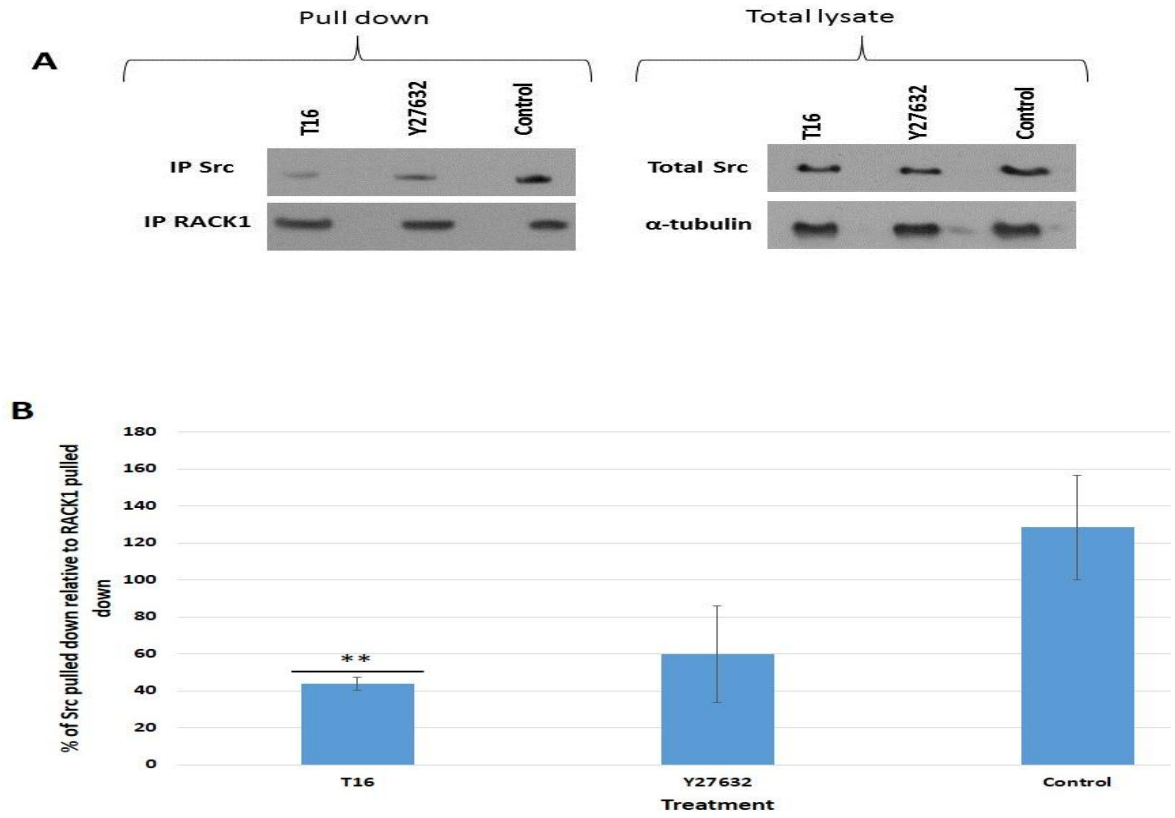
5.6.3 T16 treated hPSC have less intracellular Src-kinases bound to RACK1

Considering the lack of kinase inhibition of T16, it is possible that it mediates its pro-survival effect by disrupting signalling complexes. To determine if RACK1 and Src-family members interact directly in hPSC and whether T16 disrupts this relationship, co-immunoprecipitations (co-IP) were performed. Untreated cells (hiPSC) as well as cells treated with T16 or Y27632 were grown to confluence and non-denaturing lysis buffer used to produce protein lysates without disrupting protein-protein interactions. Anti-RACK1 antibody was used to pull down endogenous RACK1, which should remain bound to any binding partners, and was then processed using denaturing SDS-PAGE and western blotting as described in section 2.4.7. The resulting nitrocellulose membranes were probed with antibodies against RACK1 and total Src proteins. Total lysates were used as controls (co-IP was not performed on these). Figure 5.15A shows representative immunoblots produced, confirming successful pull-down of RACK and co-IP of Src proteins in untreated cells and those treated with Y27632 or T16.

The densitometry analysis (figure 5.15B) shows that T16 treatment resulted in significantly lower levels of pulled-down Src-kinases when compared to the untreated controls ($P=<0.01$). Treatment with Y27632 also resulted in reduced Src-kinases bound to RACK1, however this did not reach significance. Figure 5.16C shows levels of total Src proteins relative to α-tubulin. This confirms that the lysates that co-IP was performed on had equal levels of Src protein and that the reduced pull down of Src-family members observed was not a result of lower levels of starting Src proteins.

These data suggest that T16 treatment either reduces the binding of src-family kinases to RACK1 (possibly via competition for the Src binding site on RACK1) or initiates signalling events that result in the release of Src-family members from the RACK1 complex. The results also suggest that treatment with Y27632 may cause the release of Src-family members from RACK1; however previous results indicate that this may not be essential in Y27632 mediated survival of hPSC.

Taken together, the data garnered in sections 5.6.1 through 5.6.3 shows that hPSC express RACK1 and that RACK1 and Src-family kinases are bound to each other in hPSC. These data also show conclusively that T16 treatment results in reduced Src-family members binding to endogenous RACK1. Furthermore, T16 treatment resulted in decreased Src-family kinases binding to a GST-RACK1 fusion protein, which suggests that there may be less freely-availably Src proteins in the cytoplasm of the T16 treated cells, possibly due to Src-family members binding to alternative intracellular proteins and thereby eliciting a specific response to T16.



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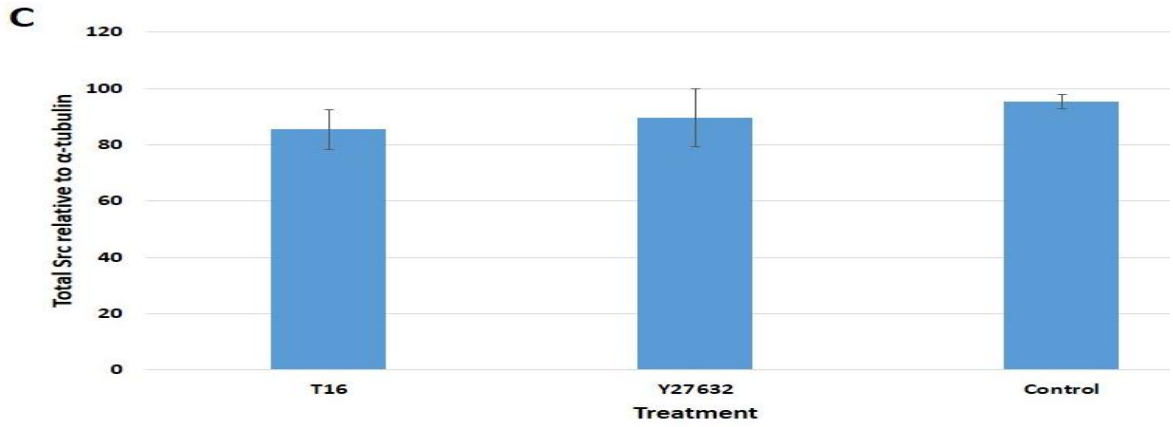


Figure 5.15- Co-IP of Src with RACK1. Untreated as well as T16 and Y27632 treated hiPSC (NMF-iPS6) were harvested and co-IPs performed using RACK1 antibody. SDS-PAGE and western blots were performed and membranes probed for Src-family kinases and RACK1. (A) Representative Immunoblots produced showing pulled down Src family kinases and RACK1 as well as Src-family kinases and α-tubulin from control lysates (no co-IP performed). (B) Shows levels of pulled down Src proteins relative to pulled down RACK1 as determined by densitometry. T16 treated cells had significantly reduced Src family kinases pulled down when compared to untreated cells (P=<0.01). (C) Control lysate Src proteins relative to α-tubulin as determined by densitometry. This shows that there were equal volumes of Src in each lysate that co-IP was performed on. Data shown as the mean value ± SEM, n=3.

5.7 Summary

Presented within this chapter are data that further elucidate the mechanism through which T16 mediates its pro-survival effect on hPSC. It has been shown that hPSC express a wide range of adhesion related genes including many α and ß integrin sub-units, cadherins and Src-family kinases. The data shows that T16 does not upregulate expression of any of these genes at the transcriptional level, and had no effect on any integrins tested at the protein level. A number of pathways have been shown to have critical roles in the apoptotic response to dissociation in hPSC such as PKC signalling which appears to be pro-apoptotic, and Pi3K signalling which seems to have a protective effect in hPSC as determined by kinase inhibition studies. The greatest difference between treatment types was observed in the response of hPSC to the Src family inhibitor PP2, which completely ablated the pro-survival effect seen in response to T16 treatment, but had no effect on Y27632 treated cells. This detrimental effect of PP2 is time-dependent, with Src-family kinase activity being essential immediately following dissociation, but being dispensable once the cells have formed cell-matrix adhesions. T16 treatment does not lead to increased phosphorylation of endogenous Src-family kinase within hPSC, however it may

alter the relationship between Src-family kinase and binding partners. The data produced in this chapter confirms RACK1, which was most highly expressed adhesion related protein at the transcriptional level, as a binding partner for Src-family kinases in hPSC, and suggests that T16 treatment may mediate its pro-survival effect by altering this relationship and potentially by making Src more available to interact with other intracellular partners. Elucidation of this critical role of Src/Src containing signalling complexes in response to T16 would have been the priority for any additional studies had time permitted.

6 Transcriptional analysis of T16 treated HPSC in response to dissociation

6.1 General introduction

As T16 is not a kinase inhibitor and prolonged pre-treatment time (>4hrs) enhanced the pro-survival effect, it was possible that it acts via transcriptional regulation rather than post-translational modification of signalling components. In an effort to better understand the transcriptional response to dissociation and also to determine if there was any differential expression in response to treatment with T16 or Y27632, an Affymetrix based microarray experiment was designed and performed using human iPSC. To our knowledge, no previous publications have investigated the complete transcriptional response to dissociation in hPSC.

The aims of this study were to:

- Determine if T16 transcriptionally primes hPSC for survival upon dissociation

- Highlight any major pathways implicated in the T16 mediated response to dissociation

- Further characterise the transcriptional response of hPSC to enzymatic dissociation

The work described in this chapter was performed in conjunction with the Glasgow Polyomics Facility, and analysis aided by the valuable input from Dr Martin McBride, Dr John McClure and Mr Mohammed Dashti (ICAMS, University of Glasgow).

6.2 Experimental design

In order to determine whether T16 was transcriptionally 'priming' hPSC for survival and to gain a better understanding of the transcriptional response of hPSC to dissociation, an Affymetrix HuGene ST 1.0 Human Array platform based microarray experiment was designed. This experiment was designed to compare untreated hPSC to hPSC that were treated with either T16 or Y27632 over various critical time points. Cells were harvested for the first time point (designated control -24hr) 24hr prior to passage and prior to the addition of any survival compound. It is at this point that T16 is usually added to cells, so this sample would act as an untreated baseline control. The next cohort of samples was collected immediately preceding dissociation via direct on the plate lysis to avoid any effect of harvesting. Samples were taken from T16 treated (for 24hours), Y27632 treated (for 2hours) and untreated cells (24hours DMSO only). The next group of samples were harvested immediately after dissociation to single cells and following a brief wash step (<10mins post dissociation). Again samples were harvested from T16, Y27632 and untreated cells. The final cohort of samples was harvested from cells after an 8hr recovery period (on fibronectin coated wells in Stempro SFM culture medium) with their respective treatments. At this last time point there were clear differneces between treatment groups, with T16 and Y27632 treated cells being largely adherent, and untreated cells remaining mainly in suspension. There were a total of 10 samples which were collected from each of 3 separate experiments that were performed on cells from consecutive passages from hiPSC that had been mechanically maintained as described in section 2.2.1.1.Each sample was taken from a pooled collection from 2 wells at each time point to reduce variation and to include sufficient material for high quality RNA preparation. A schematic of this experiment can be seen in figure 6.1. Arrows represent the most relevant comparisons that could be made between groups.

Samples were processed for analysis as described in section 2.3.6.

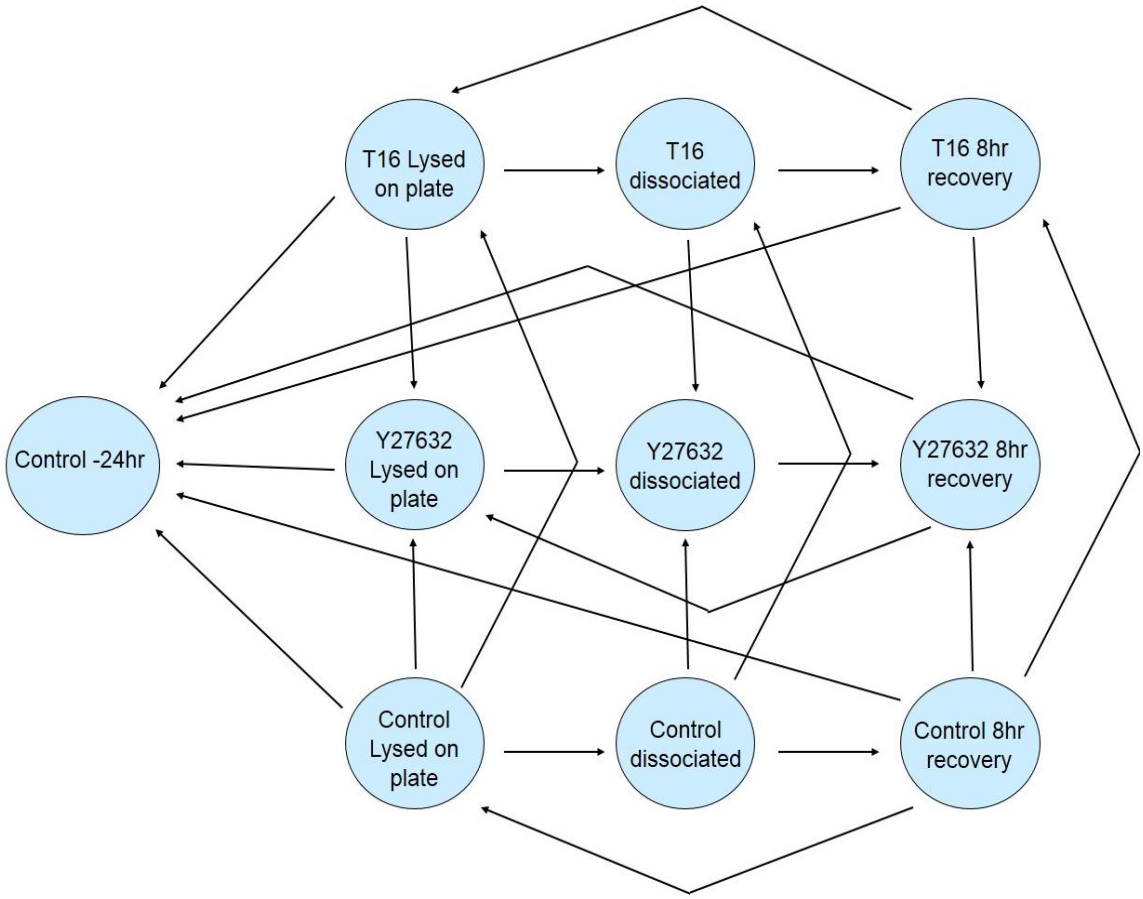


Figure 6.1- Schematic of experimental design. Samples were collected from hiPSC 24hours prior to dissociation. Untreated samples as well as T16 and Y27632 treated samples were harvested at 3 further time points. These were immediately before and after dissociation and after an 8hr recovery period. Arrows represent the comparisons that were made.

6.3 Quality control

6.3.1 HPSC were karyotypically normal at the point of sample collection

In order to ensure accurate and reproducible results, cells were analysed for cytogenetic stability prior to use. This was performed at the Medical Genetics department at Yorkhill Hospital (NHS Greater Glasgow & Clyde) as described previously. As can be seen, cells were karyotypically normal (46 XY) prior to use in this experiment (figure 6.2).

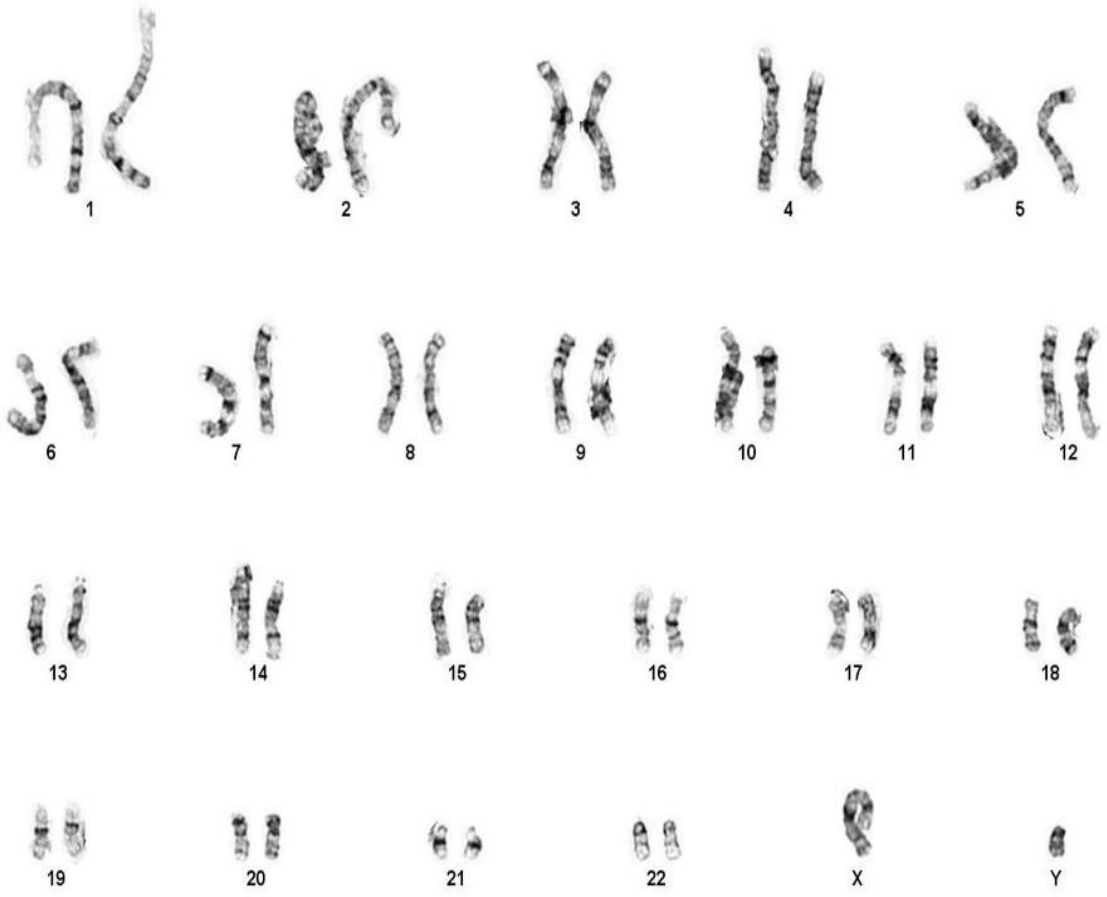


Figure 6.2- Cytogenetic stability of hPSC. Cells were analysed for cytogenetic stability prior to sample collection. Results show that cells retained a normal karyotype of 46 XY. Analysis was performed at Yorkhill Hospital (NHS Greater Glasgow & Clyde).

6.3.2 Untreated hPSC were apoptotic after 8hr recovery period

To confirm that an 8hr recovery period was sufficient to observe the various phenotypes associated with the different treatment types, i.e. untreated cells are apoptotic whilst T16 and Y27632 treated cells are not, an apoptosis assay was performed on hPSC 8hrs post dissociation. HiPSC were harvested after 8hr and analysed using the flow cytometer as described in section 2.5.1.

Cells treated with T16 or Y27632 had only 7% of late apoptotic cells (positive for both PI and Annexin V), whereas untreated cells were 76% late apoptotic. This confirms that an 8hr recovery period is sufficient time for cells to show signs of apoptosis, or to recover, and is consistent with that seen by other groups (Ohgushi *et al*, 2010).

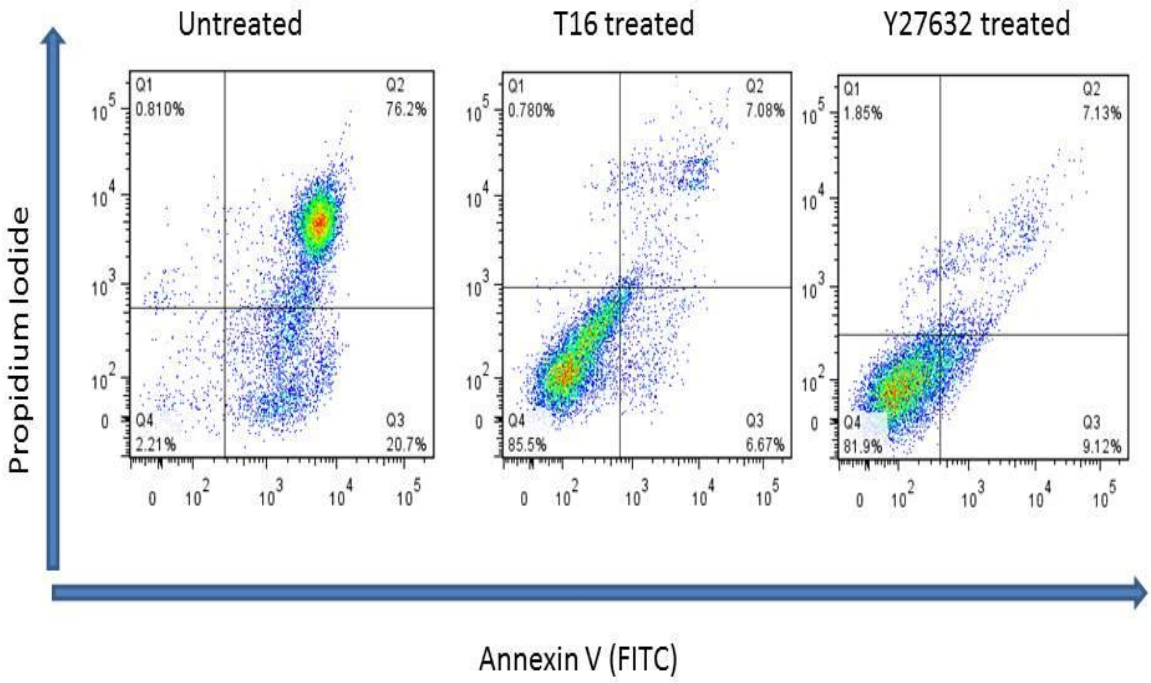


Figure 6.3- Apoptosis assays on hPSC 8hr post dissociation. HPSC were treated with T16, Y27632 or untreated and then enzymatically dissociated to single cell. Cells were replated into fibronectin coated culture plates and allowed to recover for 8hrs. Cells were harvested and analysed using flow cytometry. Data shows that the majority of untreated cells are late apoptotic (76%) whereas T16 treated and Y27632 showed only 7% and 23% of late apoptotic cells respectively.

6.3.3 HPSC expressed high levels of pluripotency markers

It was critical that the cells used to produce samples for analysis were of high quality and expressed sufficient levels of pluripotency markers. To confirm that the cells were pluripotent at the point of use, flow cytometry was performed on hPSC prior to use to confirm the expression of pluripotency and lack of differentiation markers. Samples were processed and analysed as described previously immediately following enzymatic dissociation. The pluripotency marker SSEA4 was expressed on >90% of cells and the differentiation marker SSEA1 detected on <5% in all samples (figure 6.4), confirming that hPSC cultures were pluripotent and of high quality.

6.3.4 RNA quality

Again, in order to optimise performance, the RNA used had to be of sufficiently high quality, having a RIN or RNA integrity number >7. This was assessed by running samples on the Agilent® 2100 bioanalyser as described in section 2.3.2.1. Agilent analysis not only produced RIN values, but also generated electrophoresis (figure 6.5A) and elecrtopherograms (figure 6.5B) for each sample. The clean bands and distinct peaks seen are indicative of high quality RNA, representing 18S and 28S ribosomal RNA (rRNA) that had not been degraded in any way. All 30 samples produced RIN values >9.

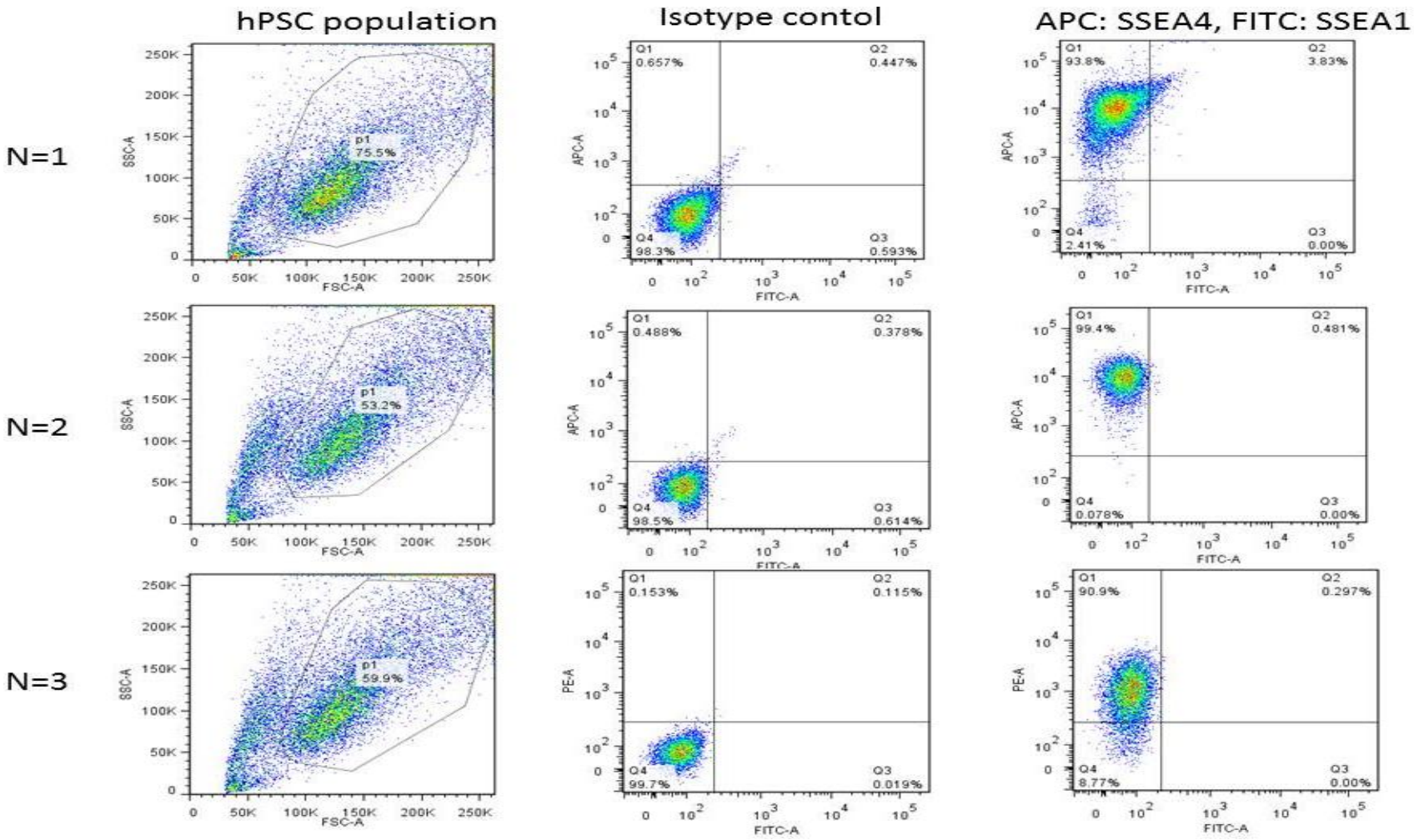


Figure 6.4- Expression of pluripotency markers on hPSC used for microarray. The expression of pluripotency and differentiation markers on hPSC (NMF-iPS6) to be used in the microarray analysis was by flow cytometry. In each of the samples the expression of the pluripotency marker SSEA4 >90% and the differentiation marker was <5%.

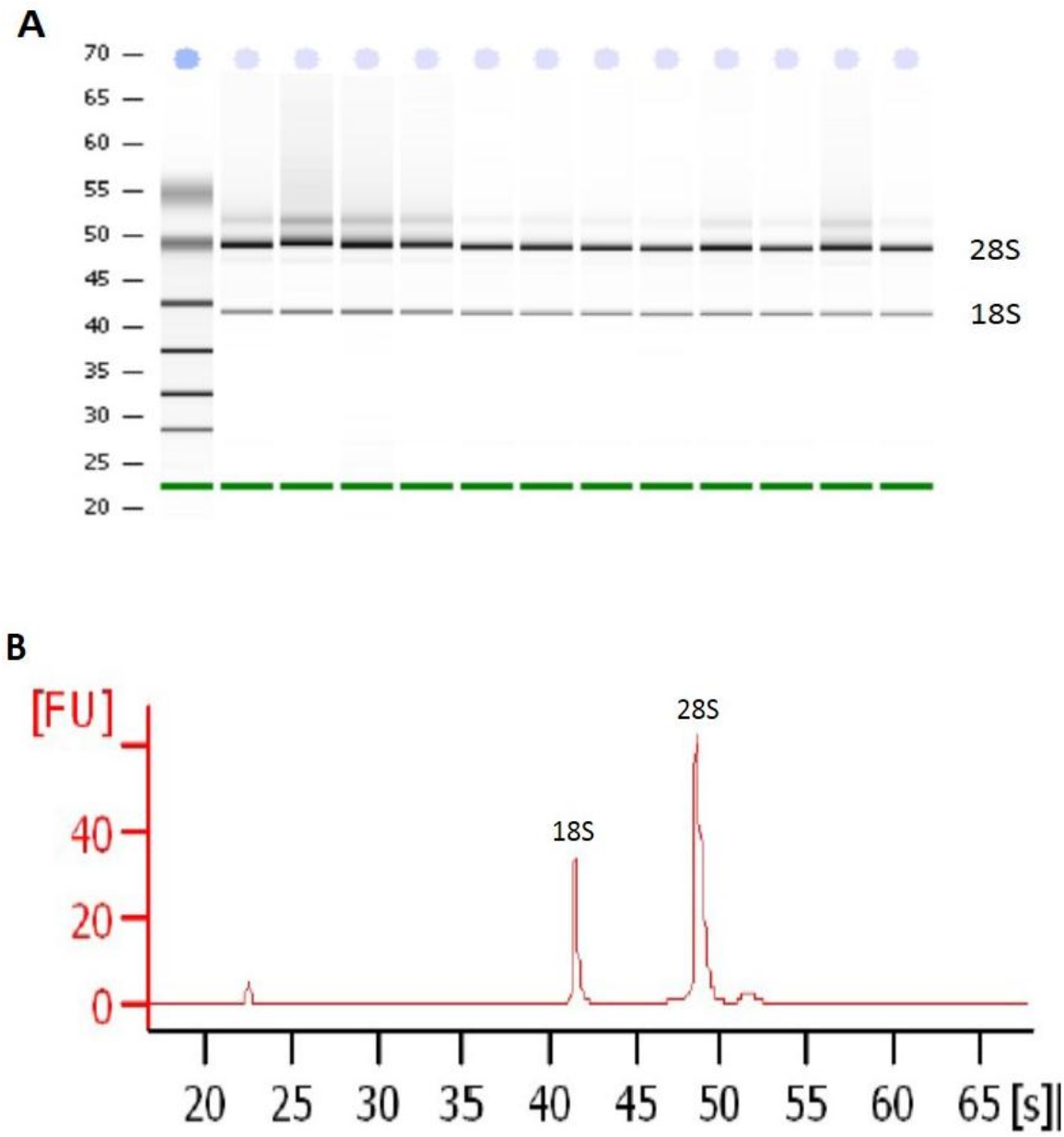


Figure 6.5- Agilent Bioanalyzer 2100 analysis of RNA quality. Each of the 30 samples to be used was subjected to analysis prior to use to confirm sufficient RNA quality. (A) Shows representative electrophoresis plots, with each column representing a single sample. 2 distinct bands can be seen in each sample representing 18S and 28S ribosomal RNA (rRNA). (B) Shows representative electropherogram from a single sample with the distinct peaks again representing 18S and 28S rRNA**.**

6.4 Data normalisation and analysis

Once the appropriate quality control steps had been performed and samples processed as described in section 2.3.6, the resulting data (in the form of .cel files) was uploaded into the Partek analysis software program. Data was normalised using the Robust Multiarray Average (RMA) method and was subject to principal component analysis (PCA). An analysis of variance (ANOVA) model was then produced which took into consideration the batch to batch variability relating to the separate chip run dates as well the separate treatments and time points. This allowed for multiple pairwise comparisons to be performed and for step-up false discovery rates (FDR) to be generated.

6.4.1 Principle component analysis

Principle component analysis is a mathematical model that performs linear transformation on the variables within the data to produce uncorrelated variables known as principal components (PCs). These PCs account for all the variability within the data set and allows samples to be visualised in PCA maps (figure 6.6). The PCA maps show that the 8hr recovery samples from all treatment groups were separate from the other conditions (figure 6.6A) and that each of the repeats were clustered sufficiently close to proceed with the analysis (figure 6.6B). From this initial analysis it was expected that the 8hr recovery samples would have many differences when compared to the other sample groups.

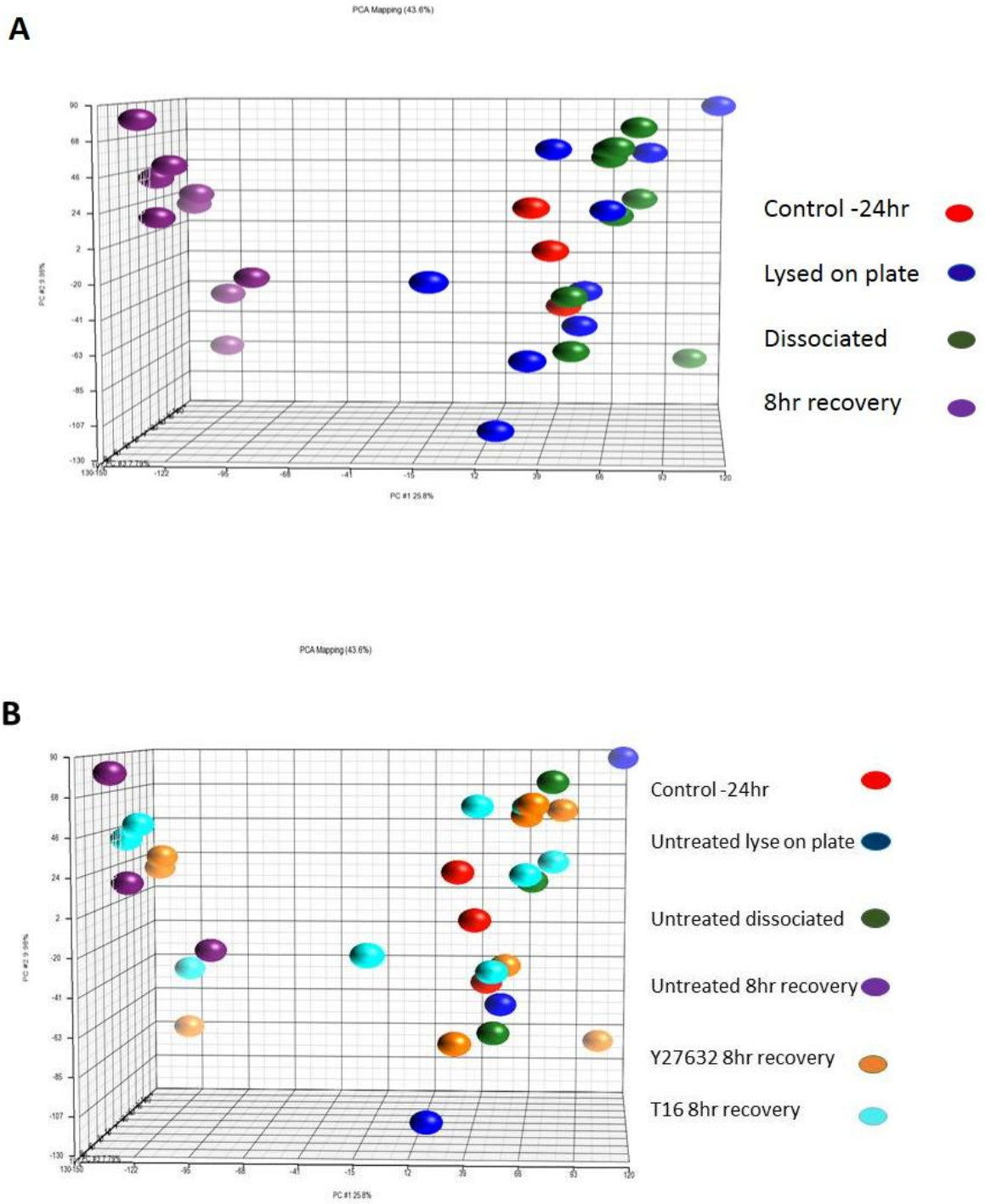


Figure 6.6- Principle component analysis. Data produced using Affymetrix HuGene ST 1.0 Human Array chips were subject to PCA. It can be seen that the cohort of samples obtained after the 8hr recovery period were distinct to the other samples (A). PCA map showing treatment type (B). Data shows the principle components for all 30 samples.

6.4.2 Ingenuity Pathway Analysis

The raw data produced by the ANOVA was uploaded and analysed by the Ingenuity Pathway Analysis (IPA) software which is able to compare the data sets to information within the Ingenuity Pathways Knowledge Base. The software produces a list of canonical pathways that are most affected by the gene changes within the data set. These canonical pathways are presented alongside a ratio which represents the number of genes in each particular pathway differentially regulated in the data set, divided by total number of genes in that pathway. Therefore, higher ratios are seen in pathways that have changes in expression in a greater number of genes within that pathway. The pathways are also presented with a P value produced using Fishers exact test right tailed which represents the likelihood that the changes in genes within the particular pathway could have resulted by chance alone.

Additionally, IPA is able to produce heat maps that give an overview of biological functions that are impacted by the changes in the data set. IPA makes predictions for each function based on its z-score and functions with a z-score ≥ 2 are predicted to be increased where as those ≤ -2 are predicted to be decreased. Functions cover a range of biological processes such as adhesion, apoptosis, cytoskeletal organisation and developmental processes.

6.5 Pairwise comparisons

As previously stated, the ANOVA model generated makes multiple pairwise comparisons between the groups and produces a list of differentially expressed probe sets between each comparison group. Only those with a FDR of <0.05 were included in the generated list of probe sets. The arrows in figure 6.1 show the large number of comparisons that can be made. However not all comparisons produced any significantly differentially expressed probes. The comparisons that produced significant differences are detailed in table 6.1.

Comparison	FDR	Differentially expressed probes
T16 Lyse on plate vs. Y27632 lyse on plate	<0.05	4
T16 8hr recovery vs. Untreated 8hr recovery	<0.05	151
Untreated 8hr recovery vs. Y27632 8hr recovery	<0.05	1900
T16 8hr recovery vs. Y27632 8hr recovery	<0.05	52
Untreated dissociated vs. -24hr control	<0.05	7
T16 dissociated vs. -24hr control	<0.05	8
Y27632 dissociated vs. -24hr control	<0.05	18
Untreated 8hr recovery vs. untreated lysed on plate	<0.05	7251
T16 8hr recovery vs. T16 lysed on plate	<0.05	7616
Y27632 8hr recovery vs. Y27632 lysed on plate	<0.05	7711
Untreated 8hr recovery vs. Untreated dissociated	<0.05	8594
T16 8hr recovery vs. T16 dissociated	<0.05	8254
Y27632 8hr recovery vs. Y27632 dissociated	<0.05	9379

Table 6.1- Differentially expressed probes.. Data shows those comparisons which resulted in differentially expressed probe sets. Only probes with an FDR of <0.05 are shown.

6.5.1 T16 treatment does not alter gene expression prior to dissociation

Given that T16 treatment requires 24hr pre-treatment to produce optimal cell survival, it was thought that it may be exerting its effect via transcriptional regulation of genes important to cell survival. The comparison between T16 treated cells pre dissociation (lysed on plate) and the -24hr control sample showed no significant changes suggesting that there was no transcriptional effect of T16. Additionally, there were no significant differences between hPSC treated with T16 and the untreated control cells when lysed prior to dissociation (table 6.1), again showing that it was unlikely that T16 altered gene expression to make the cells more resistant to dissociation.

The samples collected immediately following dissociation were compared to those collected prior to dissociation to determine if T16 prevented an immediate up or down regulation of any genes which may be associated with the process of dissociation and harvesting, however there were no significant differences between these groups regardless of treatment type. The dissociated samples were also compared to the -24hr control sample and resulted in 7, 8 and 18 differentially regulated probes for untreated, T16 and Y27632 treated cells respectively. None of the changes were related to genes of interest and had no common relationship when uploaded onto IPA. Furthermore, the few changes seen were very small with the highest change in expression in any of these probes being only a 1.7 fold increase.

The highest number of changes in expression were seen in the comparison of hPSC after the 8hr recovery period to cells immediately pre or post dissociation, with comparisons between these samples having >7000 differentially expressed probes (i.e T16 8hr recovery vs T16 lysed on plate). The comparison between the 8hr recovery samples and the dissociated cells had the greatest number of changes and was therefore the focus for subsequent analysis. This comparison group should encompass all the relevant biological changes related to the dissociation and recovery processes given that there were no changes between -24hr control cells and the lysed on the plate samples (therefore no probes would be missed). There were also differentially expressed probes between T16, Y27632 and untreated hPSC at the 8hr recovery point and these differences were

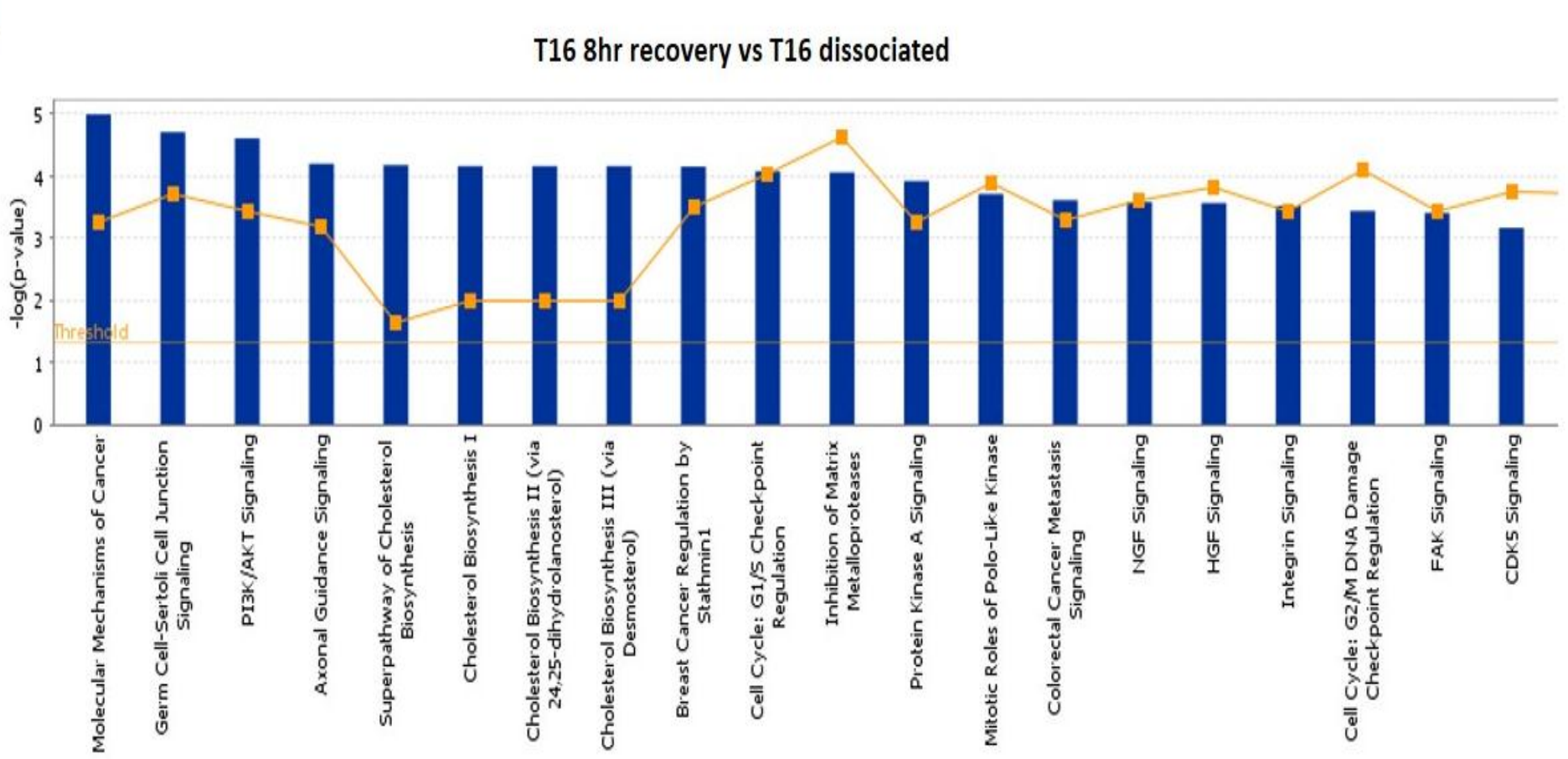
also further investigated (i.e T16 8hr recovery vs Control 8hr recovery) as they may reveal gene changes related to the pro-survival effects of either or both molecules.

Although there were significant changes detected between comparison groups, the fold changes observed were surprisingly small regardless of the comparisons being made. For example the highest fold change observed within the entire data set was between T16 treated 8hr recovery cells and T16 treated cells post dissociation cells, where a 16-fold increase in TRIM43 (tripartite motif containing protein 43) was detected. However, the vast majority of changes within each of the comparison groups were <1.5 fold. With such small fold changes, it was predicted that it might be difficult to successfully validate any targets of interest, however many small changes within a single pathway could still lead to a significant modification of the activity of that pathway.

6.6 Canonical pathway analysis

As previously stated, IPA creates a list of canonical pathways that the data sets are impacting. Data sets were uploaded to IPA to compare the 8hr recovery cells to the cells immediately following dissociation for each treatment type. It was notable that although there are a number of pathways that are present in the top 20 pathways influenced by each treatment, there are also a number of pathways that are only present in one or two treatment types (Figure 6.7). The top canonical pathway in T16 treated cells was the molecular mechanisms of cancer; however this particular pathway is also highly affected in Y27632 and untreated cells, showing as the 5^{th} and 2^{nd} pathways respectively. The top pathway in Y27632 treated cells was the protein kinase A (PKA) pathway, which was again in the top 20 pathways for T16 and untreated cells. The super pathway of cholesterol biosynthesis was the top canonical pathway in untreated cells. However, cholesterol biosynthesis was also common to T16 and Y27632 treated cells. We therefore focussed on pathways that were not common to all three to highlight pathways that may be differentially regulated in and thus important in the response of hPSC to dissociation.

A



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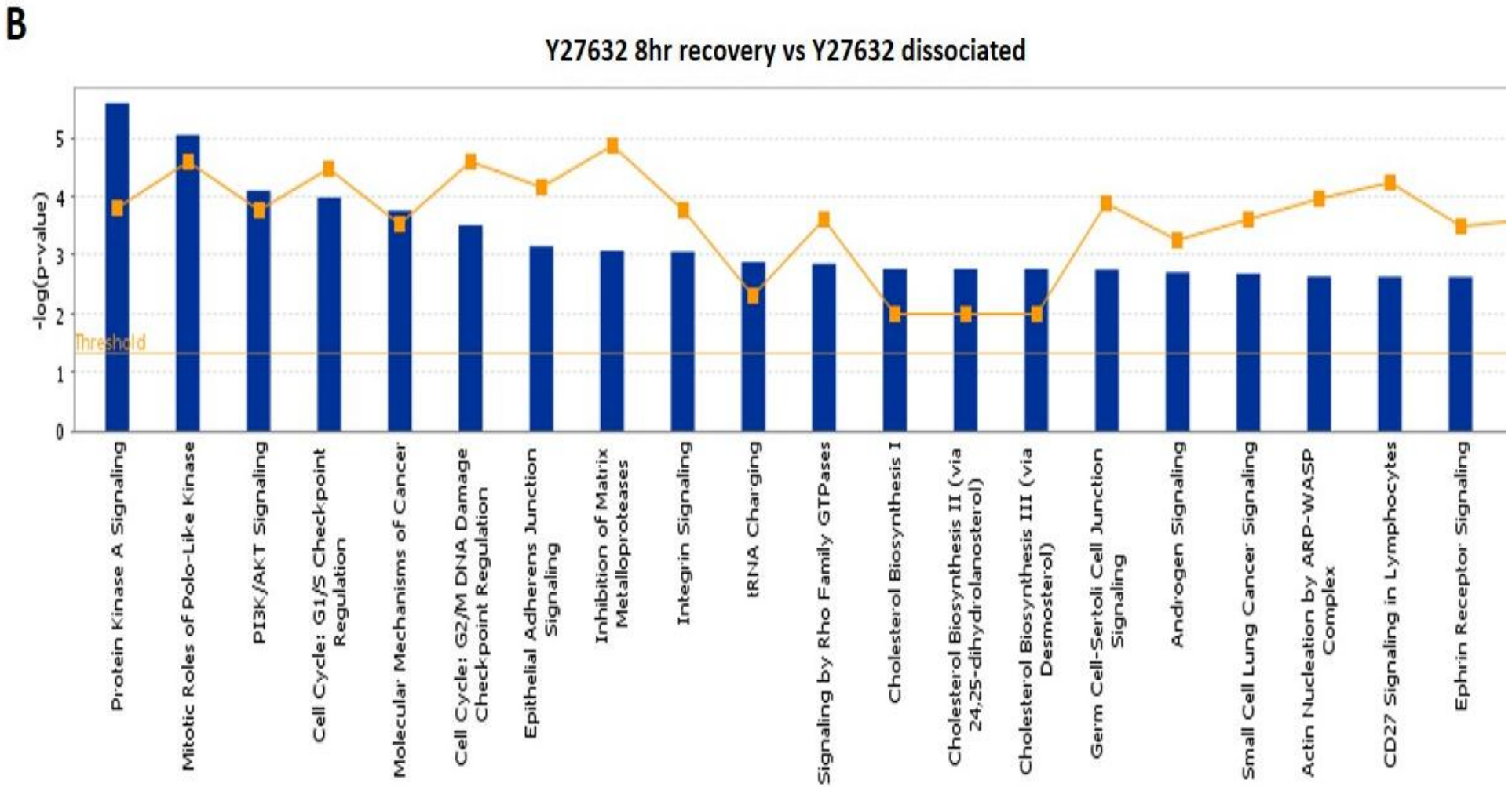

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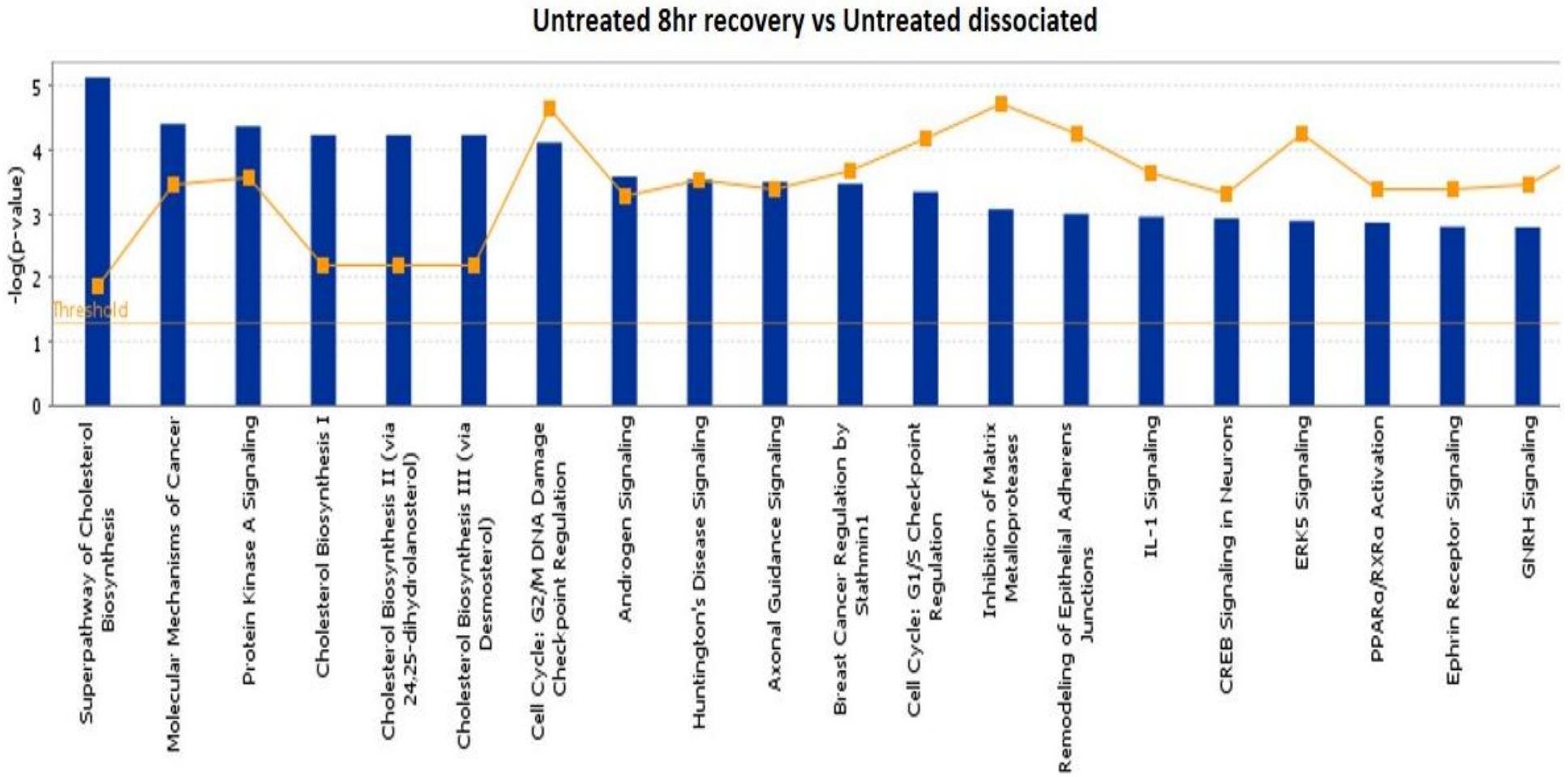


Figure 6.7- Canonical pathway comparison. IPA was used to compare the canonical pathways altered in response to T16 (A), Y27632 (B) and no treatment (C). Data shows the top 20 pathways for each comparison group. The yellow line shows the ratio which represents the number of genes in each particular pathway differentially regulated in the data sets, divided by total number of genes in that pathway.

6.6.1 Pi3k/Akt signalling pathway

The first canonical pathway that may be of relevance is the Pi3K/Akt signalling pathway, which was the 3rd most affected canonical pathway in both T16 and Y27632 treated cells whereas it does not appear in the top 20 canonical pathways in untreated cells. This is particularly relevant given that Pi3k signalling has a known role in cell survival (Eiselleova et al, 2009) and that previous studies within this thesis indicated that inhibition of Pi3K signalling reduces cell survival in hPSC (Figure 5.8). Although no change in expression was greater than ± 4-fold within this pathway (with the majority being <2), the canonical pathways generated by IPA show that treatment with T16 or Y27632 resulted in differential expression in a large proportion of genes within the Pi3K/Akt signalling pathway (Figure 6.9). The data showed that there was upregulation of several Pi3K family members which could be activated in response to integrin activation or downstream of receptor tyrosine kinases (RTK) such as FGFR. PiK3AP1 (phosphoinositide 3 kinase adapter protein 1) and PiK3R1 (phosphoinositide 3 kinase regulatory sub-unit) which can function together to activate Pi3K signalling were both upregulated in the 8hr recovery cells in response to T16 treatment when compared to the dissociated cells (1.5 and 1.9 fold respectively) but not in untreated cells. PiK3AP1 was also upregulated in Y27632 treated cells in the 8hr recovery cells (1.4 fold) but Pi3KR1 was not.

In addition to this, the expression of another family member, PiK3C2B (Phosphatidylinositol-4-Phosphate 3-Kinase), was upregulated in both T16 and Y27632 treated cells in the 8hr recovery time point (1.7 and 1.6 fold increase respectively), but not in the untreated cells.

The data produced also supports the link between Pi3K signalling and pluripotency. In both the Y27632 and T16 data sets, Nanog expression was increased (1.4 and 1.5 fold respectively) in the 8hr recovery cells, which may be a result of downstream Pi3K signalling which has been reported lead to Nanog activation in hPSC (Wi *et al,* 2007; Wang *et al,* 2009). This may also indicate that Nanog is only maintained in cells that successfully re-attach to the ECM post dissociation.

6.6.1.1 Validation of gene targets within the Pi3K/Akt pathway

In order to validate selected targets from the Pi3K/Akt pathway, qRT-PCR analysis was performed using the RNA extracted from the original samples. PiK3R1, PiK3AP1 and PiK3C2B were chosen as suitable targets for validation. Results from the validation (figure 6.8) show that although there were statistically significant increases (P=<0.05) between the time points for PiK3R1 and PiK3AP1, the increases were not treatment specific, with all treatments resulting in upregulation in these genes, even the untreated cells in which ~80% are undergoing apoptosis.

When validating the changes in expression of PiK3C2B, none of the original changes observed were validated using qRT-PCR, with no significant difference in expression observed between the treatments or time points.

A complete summary of validation targets can be seen in table 6.2.

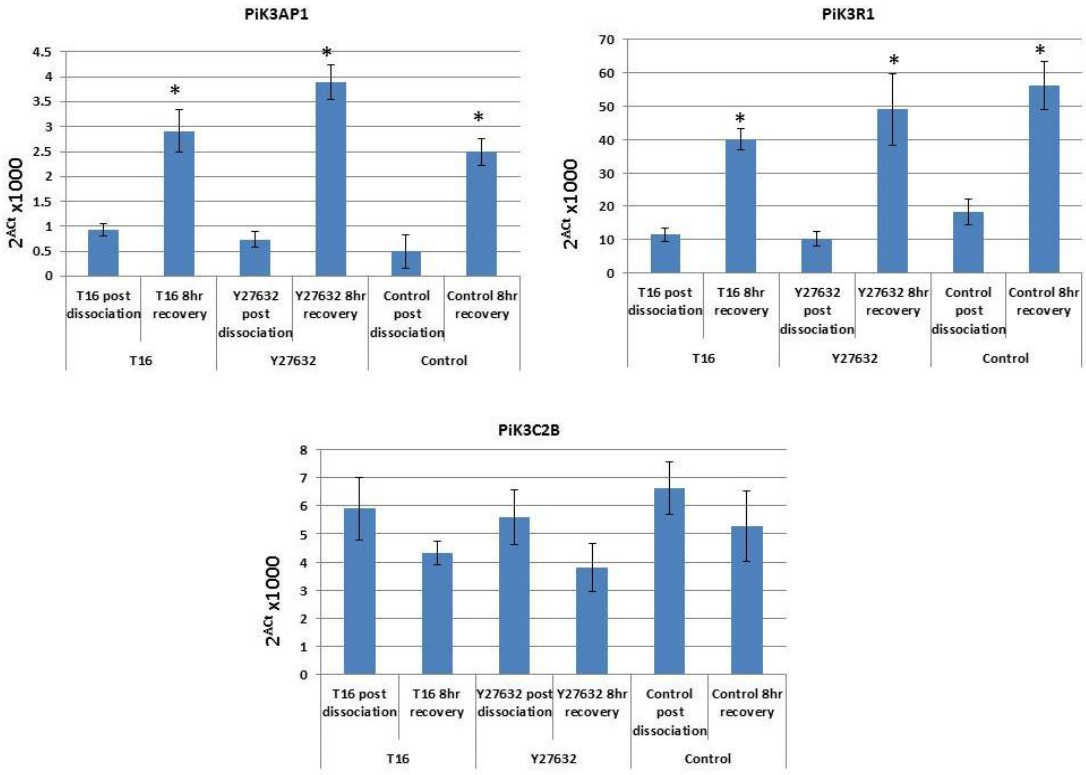
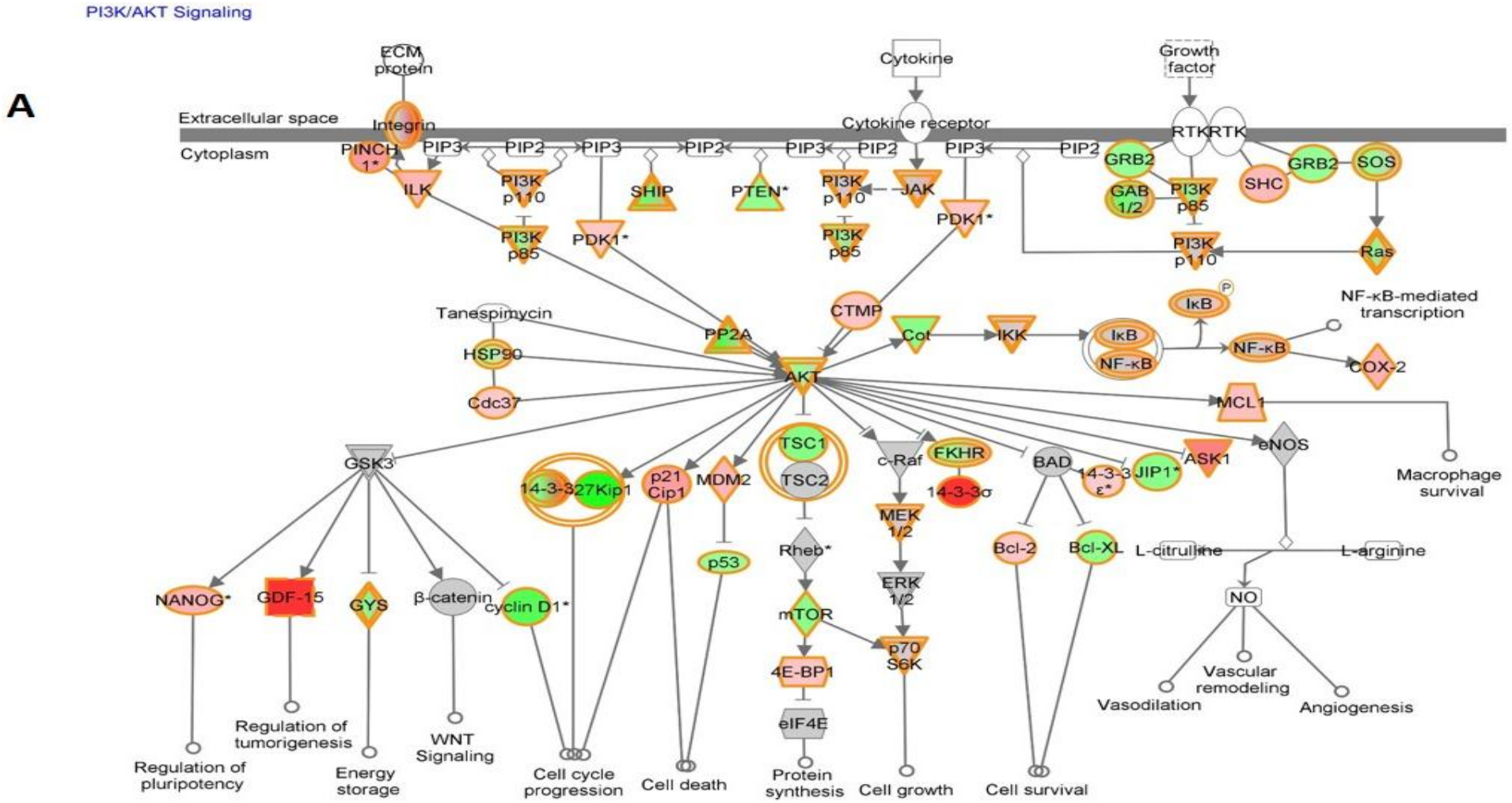


Figure 6.8- Validation of Pi3k/akt target genes. Several members of Pi3K/Akt signalling pathway were selected for validation. Targets were validated using the original RNA from the array by qRT-PCR. Data shows significant increase (P=<0.05) in expression of PiK3AP1 and PiK3R1 in the 8hr recovery cells compared to cells immediately post dissociation in each of the treatment types. There were no significant differences observed in the expression of Pik3C2B. Data shown as mean $2^{\Delta Ct}$ x1000 ± SEM. Experiments were performed with technical and biological triplicates (n=3 in triplicate).



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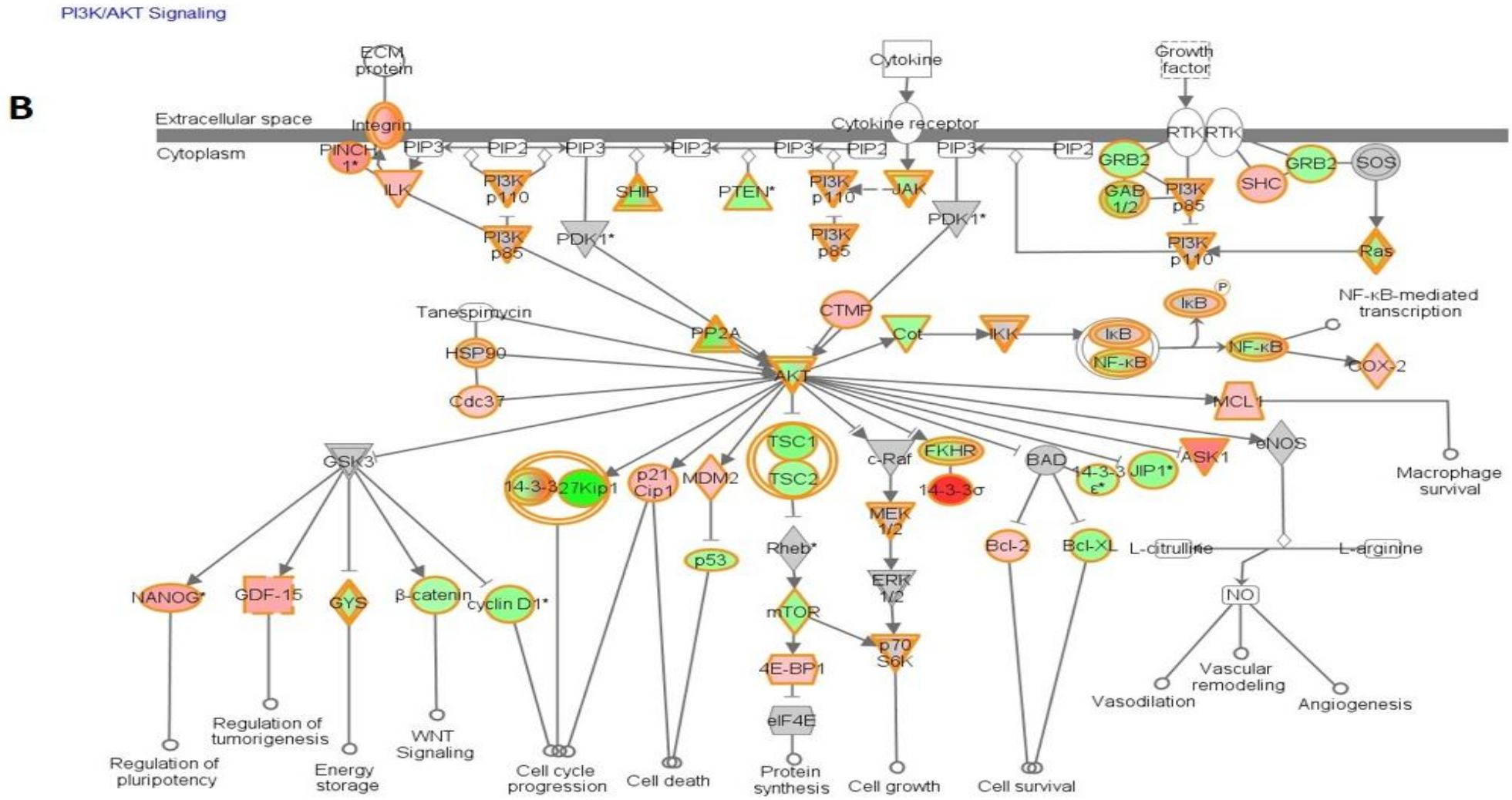


Figure 6.9- Pi3K signalling canonical pathways. (A) The Pi3K signalling pathway showing genes that were upregulated (red) or downregulated (green) in T16 8hr recovery cells when compared to T16 dissociated cells. (B) Shows the same pathway in response to Y27632 treatment.

6.6.2 Integrin signalling

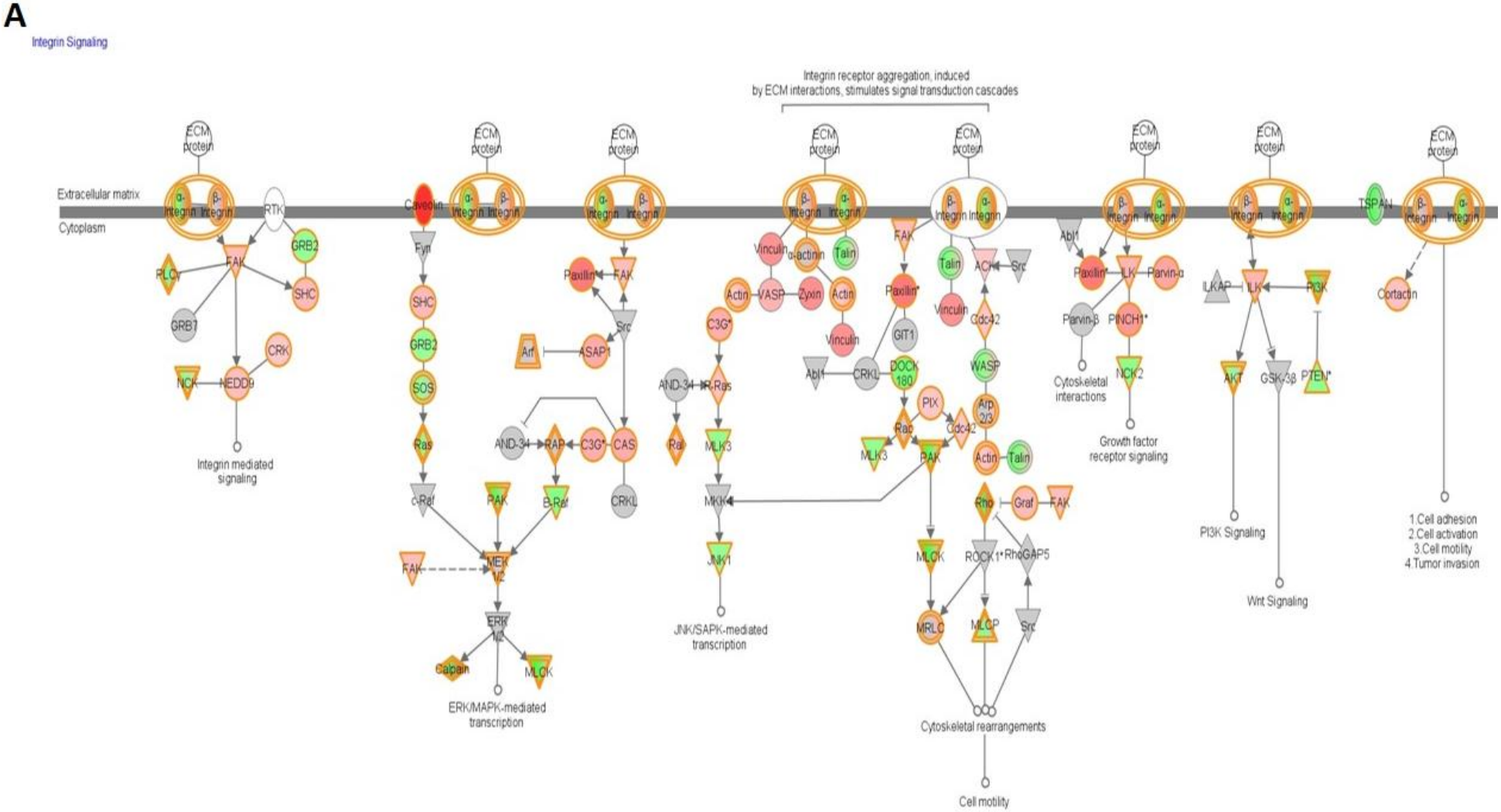
A further canonical pathway of interest was the integrin signalling pathway, which was in the top 20 canonical pathways of both T16 and Y27632 treated cells. Again, as seen with Pi3K/Akt signalling, integrin signalling was not one of the top 20 pathways affected in untreated cells and could therefore be part of the pro-survival response seen with both small molecules. The canonical pathways produced by IPA (figure 6.10) show that a large fraction of the genes within the pathway were differentially expressed in cells treated with either T16 or Y27632. This was perhaps unsurprising given that during the 8hr recovery period the hPSC are actively adhering and spreading on the ECM in response to T16 or Y27632 treatment, whereas the untreated cells mostly stay in suspension and begin to undergo apoptosis. Genes differentially regulated in response to Y27632 or T16 treatment included the integrin sub-units α6 and ß1 as well as components of focal adhesions such as FAK and paxillin. Again, although statistically significant, all of the fold changes observed were <3-fold.

These results highlight the importance of integrin signalling in response to dissociation and clearly show that hPSC are transcriptionally regulating many components of the integrin signalling pathway in response to dissociation and subsequent re-adherence. Given the adhesion dependent nature of T16 mediated survival, a number of these targets were of great interest, such as the aforementioned integrins and components of focal adhesions and were subsequently validated.

6.6.2.1 Integrin signalling pathway validation

As before, selected targets were validated using the RNA used during the original analysis. The results generated show that there were significant changes in these validation targets between the time points, but again show that the changes were not specific to treatment type. Figure 6.11 shows that all the 8hr recovery cells had significantly increased expression ($P<0.05$) of ITGA6 (integrin α6), ITGB1 (integrin ß1), PTK2 (FAK) and PXN (paxillin) when compared to the post dissociation cells. The fold changes were 2-3fold for ITGA6, ITGB1 and PTK2, and between 6-7fold increases in PXN. A summary of validation targets can be seen in table 6.2.

A



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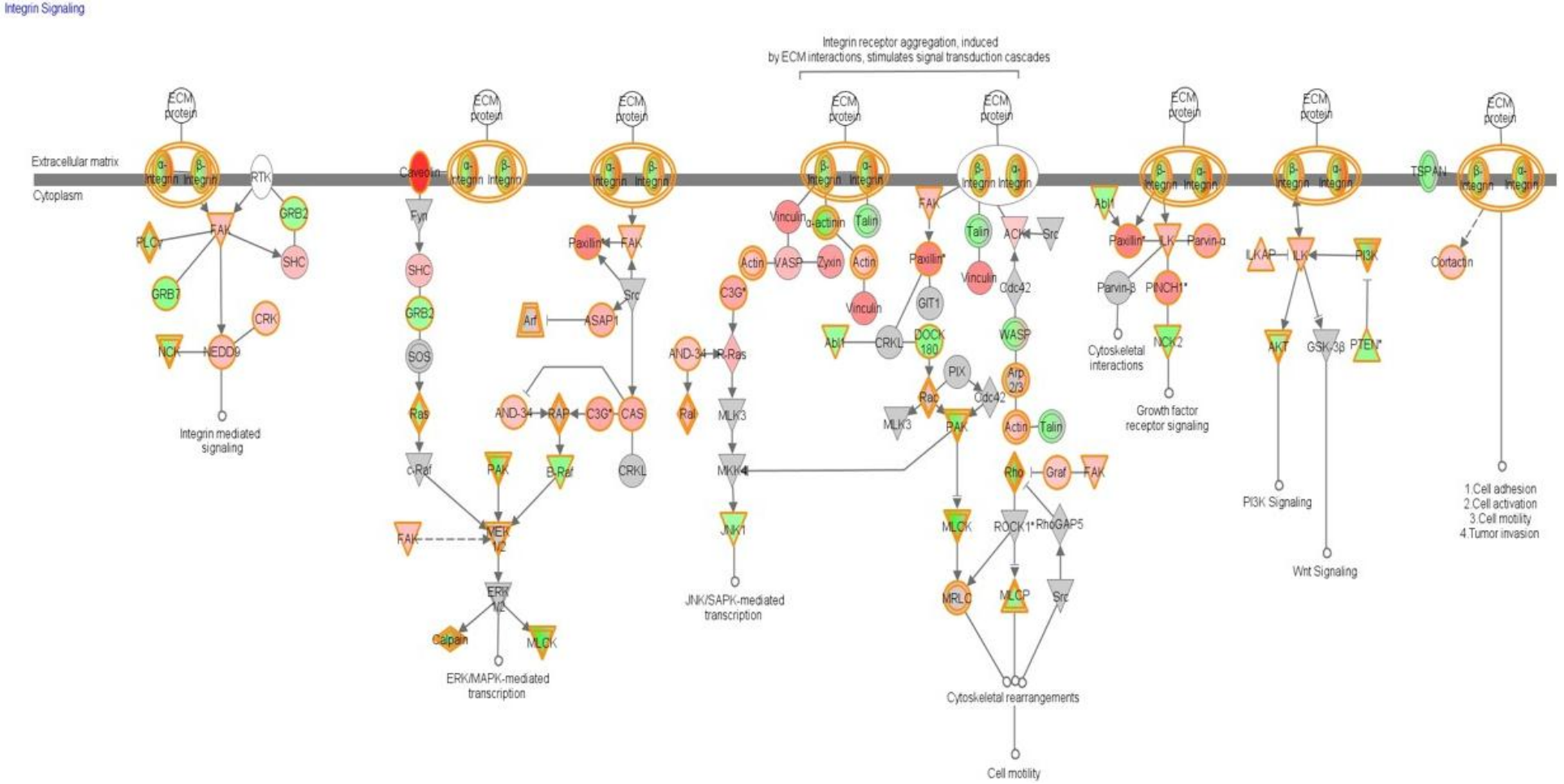

Figure 6.10- Integrin signalling canonical pathways. (A) The integrin signalling pathway showing genes that were upregulated (red) or downregulated (green) in T16 8hr recovery cells when compared to T16 dissociated cells. (B) Shows the same signalling pathway in response to Y27632 treatment at the same time points.

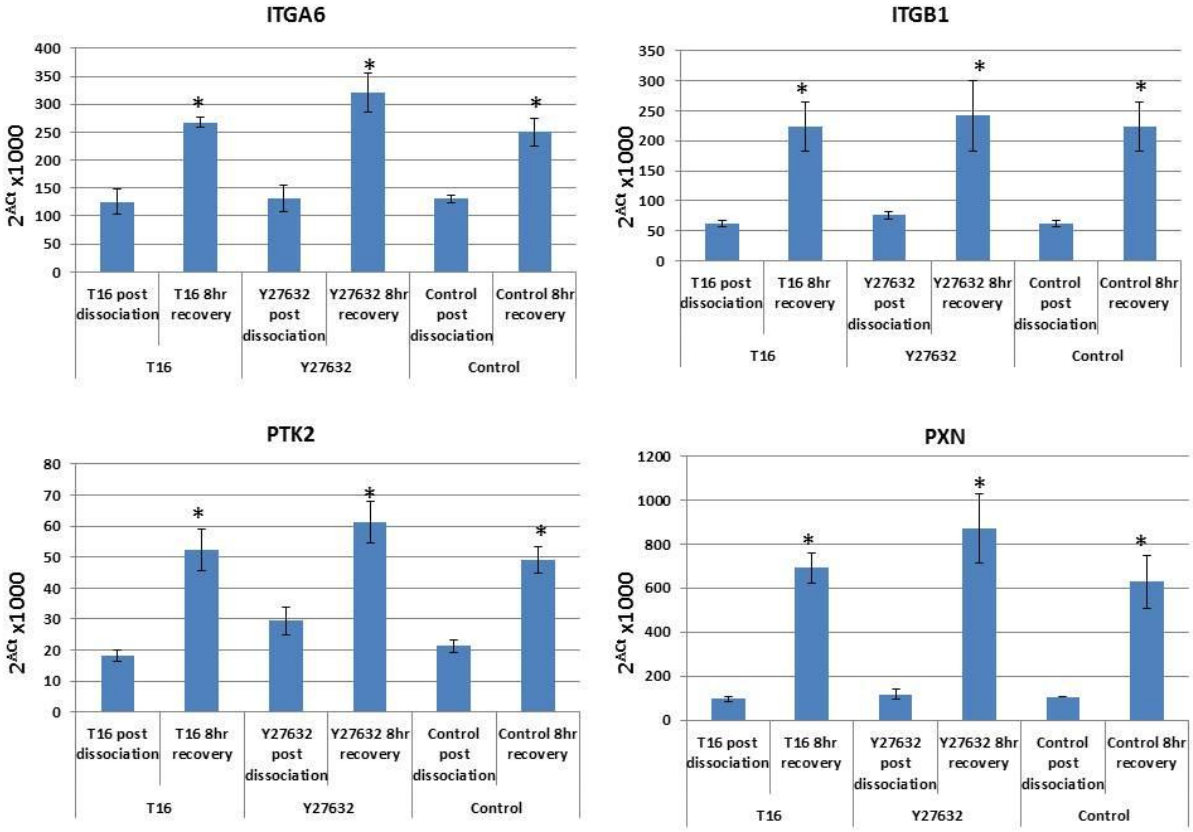

Figure 6.11- Validation of integrin signalling pathway targets. Several members of the integrin signalling pathway were selected for validation. Targets were validated using the original RNA from the array by qRT-PCR. Data shows significant increase (P=<0.05) in expression of ITGA6, ITGB1, PTK2 and PXN in the 8hr recovery cells compared to cells immediately post dissociation in each of the treatment types. Data shown as mean $2^{\Delta Ct}$ x1000 ± SEM. Experiments were performed with technical and biological triplicates (n=3 in triplicate).

6.7 Function changes

IPA software also generated a list of function it predicts to be increased or decreased as a result of the gene changes within the data set. As described in section 6.4.2, only functions that had a sufficient z-score were listed as changed. IPA predicted a total of 101 functions to be increased between the three treatment groups when the 8hr recovery cells were compared to the post dissociation cells. In order to see which functions were shared between the treatment types, a Venn diagram was produced (figure 6.12A), which showed that ~20% of the functions were shared between the treatment groups. Of those functions unique to each treatment, a number were related to cellular shape change such as the organisation of cytoskeleton and morphology of cells, and consequently shared many of the differentially expressed probes including some of those already observed within the integrin signalling pathway as discussed

above. Interestingly, T16 treated cells were predicted to have increased cell viability, whereas Y27632 treated cells were predicted to have increased attachment of cells. IPA predicted increased cell survival in each treatment group, however, the z-score was lowest for untreated cells (2.164) and highest in T16 treated cells (2.532), with the z-score for Y27632 treated cells being 2.238, which suggest there is a stronger link between the gene changes observed and increased survival in T16 treated cells. Another interesting function was increased self-renewal of ESC lines, which was shared between T16 and Y27632 treated cells but not the untreated cells. This again may be due to the increased activation of the Pi3K/Akt signalling pathway and in particular Nanog activation, or may be indicative that only those cells that successfully re-attach maintain Nanog expression.

There were less overall functions predicted to be decreased between these data sets (16 in total) however, all 5 functions shared by T16 and Y27632 were relating to cell death and apoptosis, which would result in increased cell survival (figure 6.12B). Interestingly, T16 and untreated cells shared anoikis as a function that was predicted to be decreased. Anoikis is a potential mechanism through which dissociated hPSC can evade apoptosis and it appears untreated and T16 treated cells are regulating genes with known roles in this process in response to dissociation.

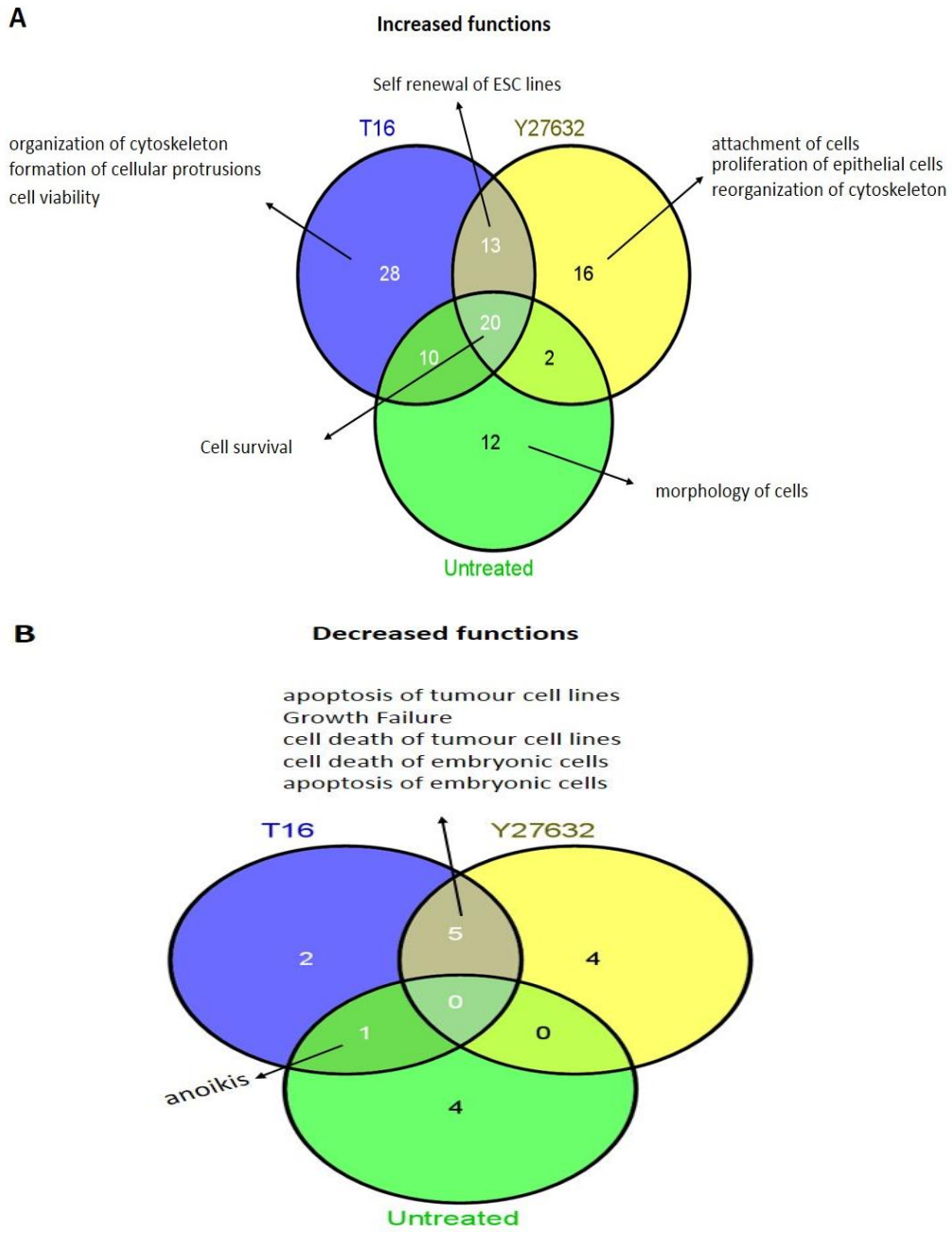

Figure 6.12- Predicted function changes between treatments. (A) Venn diagram showing functions predicted to be increased in each data set. Samples shown are 8hr recovery cells compared to dissociated cells with after each treatment. Functions of interest are listed outside of diagram (B) Venn diagram showing the functions predicted to be decreased based on the data sets.

6.7.1 Validation of functions relating to apoptosis

The most interesting functions were those which suggesting decreased apoptosis or increased cell survival, so a number of genes within these functions were validated. From within the functions shared between T16 and Y27632 treatments

that predicted decreased apoptosis, FGF2, MAP2K6 (mitogen activated protein kinase 6), SPP1 (secreted phosphoprotein 1) and ADAMTS1 (ADAM metallopeptidase with thrombospondin type 1 motif) were chosen for validation. Figure 6.13A shows that both ADAMTS1 and SPP1 had significantly increased expression in the 8hr recovery cells when compared to the post dissociation cells (P=<0.05), however, as observed with other validation targets, this was not treatment specific. Although the differences were not significant, the fold increases observed were lower in untreated cells than in T16 or Y27632 treated cells for both ADAMTS1 and SPP1. T16 and Y27632 treated cells had ~13 fold increase in ADAMTS1 compared to ~6 in untreated cells. The fold increase in SPP1 was ~31 fold in T16 and Y27632 treated cells and ~20 fold in untreated cells. The changes observed in FGF2 and MAP2K6 were not reproduced during the validation process.

In addition, as anoikis is a potential mechanism through which T16 could be mediating the pro-survival effect, genes from that pathway were also chosen for validation, these were CDCP1 (CUB domain containing protein 1), BMF (Bcl2 modifying factor), CDH1 (E-cadherin) and TGFB1 (transforming growth factor β1). The changes in expression observed in CDCP1, BMF and CDH1 from the array were reproduced during the validation process, with 8hr recovery cells having significantly increased expression of CDCP1 and CDH1 and significantly reduced BMF when compared to the post dissociated cells (P=<0.05), but again these were not specific to treatment type. When analysed by qRT-PCR the scale of change (fold change) was slightly higher than that detected during the array, which may suggest difference in sensitivity between procedures. For example, in the original array, CDH1 was predicted to be increased by 2.5-fold in T16 8hr recovery cells, however the fold change when validated showed an 11.8-fold increase. The changes seen in TGFB1 expression did not validate as there was no significant difference in expression between treatments or over time (Figure 6.13B).

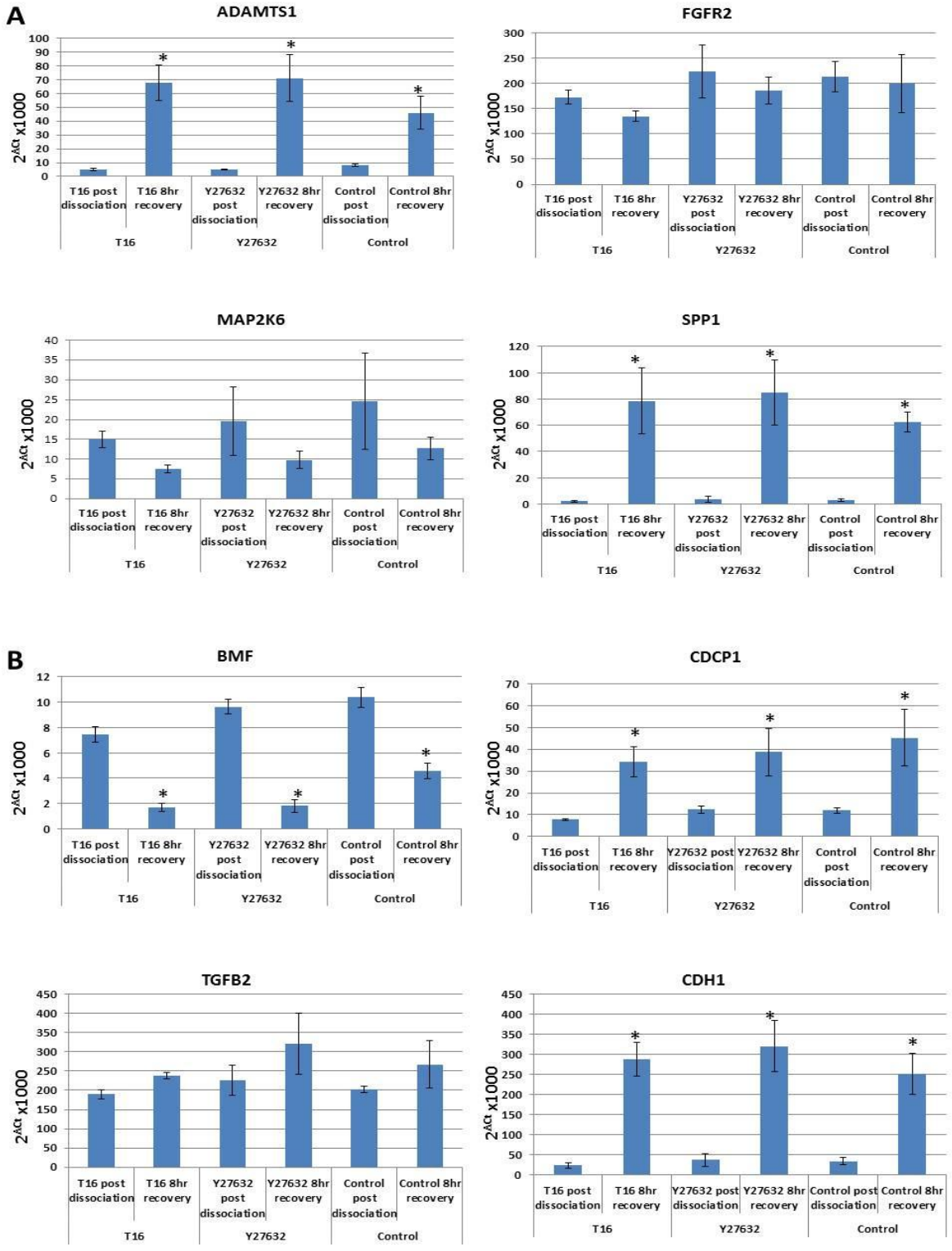

Figure 6.13- Validation of function changes. IPA predicted several functions to be either increased or decreased in response to each of the data sets. (A) From the functions predicted to decrease apoptosis shared by T16 and Y277632 treatments, there were significant increases in expression of both ADAMTS1 and SPP1 in the 8hr recovery cells compared to the post dissociation cells ($P<0.05$), however this was also the case in untreated cells. The changes in expression in FGF2 and MAP2K6 did not validate. (B) Genes relating to the function of decreased anoikis were validated and show significant decrease in BMF expression in all 8hr recovery samples. CDCP1 expression was significantly increased in all treatment types in the 8hr recovery cells when compared to post dissociation cells ($P=<0.05$). Data shown as mean $2^{\Delta Ct}$ x1000 ± SEM. Experiments were performed with technical and biological triplicates (n=3 in triplicate).

6.8 Gene expression unique to T16

Although there were a number of canonical pathways and functions that were predicted to be altered in these data sets, the gene changes that were unique to the T16 8hr recovery cells had yet to be investigated. Using the raw data file produced from the ANOVA, all significantly differentially expressed genes with fold changes greater than 1.2 or less than -1.2 were used to compile the Venn diagram below (figure 6.14). The data show that ~26% of differentially expressed genes were common to all three treatments and that a further 30% were shared between T16 and untreated cells. However there were 1009, 1098 and 650 genes that were unique to T16, untreated or Y27632 treated cells respectively. From the cohort of probe targets unique to T16 treated cells at the 8hr recovery period, a number had known roles in cell adhesion and cell survival; AMIGO2 (adhesion molecule with Ig-like domain), ANGPT1 (angiopoietin 1) and CYR61 (cysteine rich angiogenic inducer 61). Each of these genes was significantly upregulated in T16 treated hPSC in the 8hr recovery cells when compared to the dissociated cells, with relatively large fold changes of 4.6, 2.6 and 3.8 fold respectively.

The changes in expression in these genes would be predicted to result in increased adhesion and survival and were therefore validated using qRT-PCR (figure 6.15). The results, as with previous validation targets, show there was significant upregulation of these genes ($P=<0.05$) in the 8hr recovery cells when compared to the cells lysed immediately following dissociation, however, these did not turn out to be unique to T16, with there being no significant difference between treatment groups.

Although these targets do not appear to be unique to T16 treated cells, these results highlight a number of genes that are clearly upregulated by hPSC in response to dissociation and may be interesting targets to investigate at the protein level.

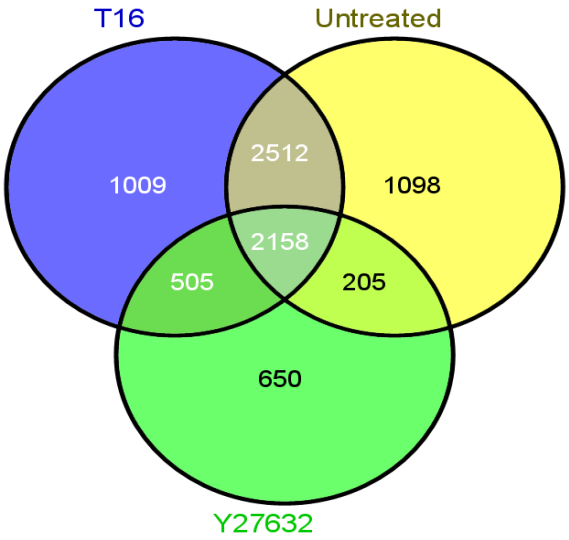

Figure 6.14- Venn diagram of gene expression after 8hr recovery period. Differentially expressed cells from 8hr recovery cells in response to each treatment type were used to create a Venn diagram showing genes unique to each treatment. Only genes with fold changes of >1.2 or <-1.2 were included.

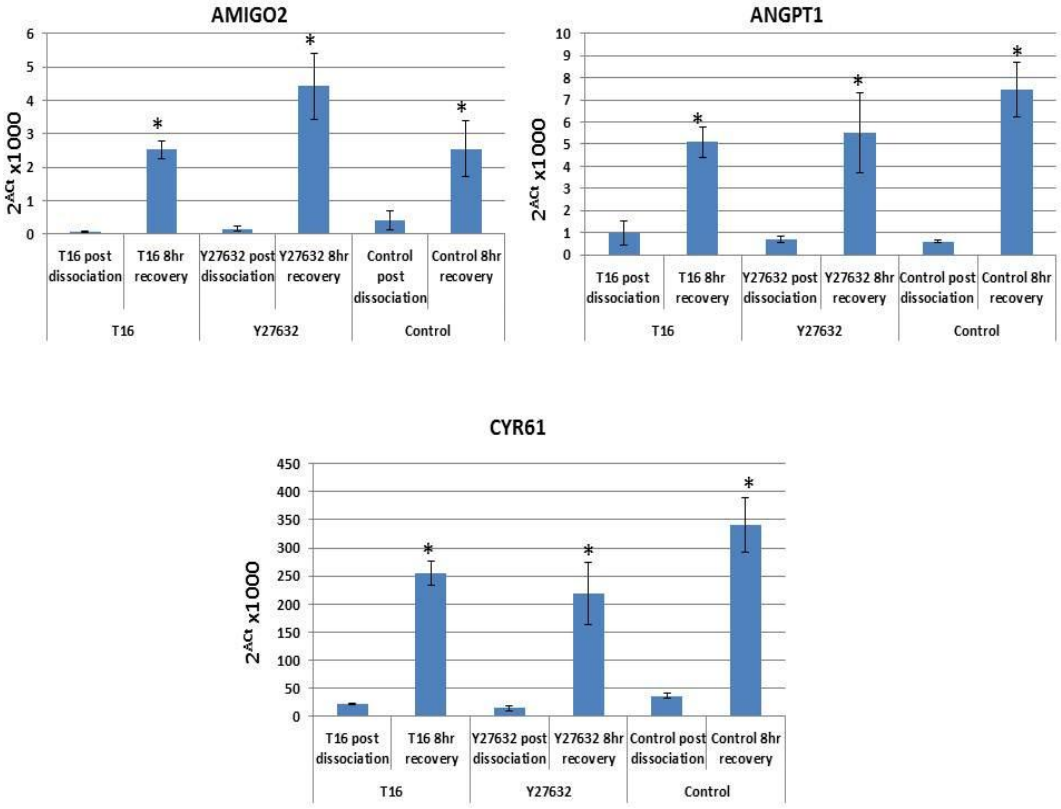

Figure 6.15- Validation of genes unique to T16 treatment in 8hr recovery hPSC. Candidate genes with high fold changes and functions relating to adhesion and cell survival which were unique to T16 treated cells were validated by qRT-PCR. The results show that although AMIGO2, ANGPT1 and CYR61 were significantly upregulated in the 8hr recovery cells when compared to dissociated cells, both Y27632 and untreated control cells also had significantly increased expression (P=<0.05). Data shown as mean $2^{\Delta Ct}$ x1000 ± SEM. Experiments were performed with technical and biological triplicates (n=3 in triplicate).

	8hr recovery vs dissociated microarray fold changes			
Gene	**T16**	**Y27632**	**Untreated**	**Validation**
PiK3AP1	1.5↑	1.4↑	n/a	Significantly increased expression in 8hr samples regardless of treatment type
PiK3R1	1.9↑	n/a	n/a	Significantly increased expression in 8hr samples regardless of treatment type
PiK3C2B	1.7↑	1.6↑	n/a	Target did not validate
ITGA6	1.2↑	n/a	n/a	Significantly increased expression in 8hr samples regardless of treatment type
ITGB1	1.6↑	1.4↑	n/a	Significantly increased expression in 8hr samples regardless of treatment type
PTK2	1.3↑	n/a	1.2↑	Significantly increased expression in 8hr samples regardless of treatment type
PXN	1.4↑	1.4↑	n/a	Significantly increased expression in 8hr samples regardless of treatment type
ADAMTS1	4.7↑	n/a	n/a	Significantly increased expression in 8hr samples regardless of treatment type
BMF	2↓	2.6↓	n/a	Significantly decreased expression in 8hr samples regardless of treatment type
CDCP1	2.3↑	n/a	n/a	Significantly increased expression in 8hr samples regardless of treatment type
CDH1	2.5↑	n/a	n/a	Significantly increased expression in 8hr samples regardless of treatment type
FGFR2	2.6↓	2.5↓	n/a	Target did not validate
MAP2K6	2.3↓	2.8↓	n/a	Target did not validate
SPP1	4.2↑	n/a	3.8↑	Significantly increased expression in 8hr samples regardless of treatment type
TGFB2	1.4↑	2.1↑	n/a	Target did not validate
AMIGO2	4.6↑	n/a	n/a	Significantly increased expression in 8hr samples regardless of treatment type
ANGPT1	2.6↑	n/a	n/a	Significantly increased expression in 8hr samples regardless of treatment type
CYR61	3.8↑	n/a	n/a	Significantly increased expression in 8hr samples regardless of treatment type

Table 6.2- Summary of validation targets. Table showing the fold changes observed in genes of interest in the initial data set alongside the validation results for 8hr recovery cells compared to dissociated cells in each treatment type.

6.9 Summary

Presented within this chapter are the results gained from an Affymetrix based microarray experiment, performed to better understand the transcriptional response of hPSC to dissociation, and to determine if T16 treatment transcriptionally primed hPSC for survival post dissociation. The data produced suggests that T16 does not mediate its pro-survival effect via transcriptionally priming cells for survival as there were no differentially expressed probes when T16 treated cells were compared to cells that had received no treatment. Furthermore these data confirm the importance of the Pi3K/Akt and integrin signalling pathways in hPSC and show that they are highly regulated pathways in the response to dissociation. The data also highlights a number of genes such as SPP1, AMIGO2 and CYR61 as potential important regulators of adhesion in hPSC.

7 Discussion

Human pluripotent stem cells (hPSC) such as hESC and hiPSC have great potential in a number of scientific fields, including developmental biology, toxicology, pathobiology and regenerative medicine. Since the initial isolation of hESC in 1998 (Thomson *et al*, 1998) and derivation of human iPSC in 2007 (Takahashi *et al*, 2007), huge strides have been made in driving these cell types towards the clinic. However, the culture of hPSC remains a limiting factor, with their sensitivity to enzymatic dissociation and dependence on the Rho-kinase (ROCK) inhibitor Y27632 being particularly troublesome for the move to larger-scale applications (Watanabe *et al*, 2007; Ohgushi *et al*, 2010).

In 2007, Watanabe and colleagues initially described that the Rho-kinase inhibitor Y27632 supported the survival of dissociated hESC (Watanabe *et al*, 2007). Since then Y27632 has become an indispensable tool in hPSC culture and manipulation. The mechanistic action of Y27632 has until recently been largely unknown, however in 2010 a number of publications implicated ROCK induced actin-myosin contractility as the cause of dissociation induced apoptosis. Y27632 treatment circumvents this by reducing the ROCK mediated hyperphosphorylation of myosin light chain 2 (MLC2) (Chen *et al,* 2010; Ohgushi *et al*, 2010; Walker *et al*, 2010).

ROCK inhibitors have proven to be incredibly useful, however there have been recent concerns that use of Y27632 may result in some undesirable outcomes. The small GTPase RhoA is a ubiquitously expressed protein involved in a number of fundamental biological processes including differentiation, cell division, apoptosis, adhesion and cytoskeletal regulation (Jaffe and Hall, 2005; Visvikis *et al*, 2010). Interference with such a diverse range of processes can interfere with normal cell functions, has been shown to decrease cell proliferative potential (Zweigerdt *et al*, 2011) and has been associated with an increased risk of transformation (Riento and Ridley, 2003; Liu *et al*, 2012). Furthermore, the inhibition of Rho signalling has been shown to have various lineage specific effects on cell differentiation; with inhibition being shown to enhance differentiation into endothelial cells, whilst preventing differentiation towards others such as haematopoietic cell lineages (Joo *et* al, 2012; Yung *et al*, 2011; Chen *et al*, 2012).

A number of studies that aimed to identify alternative pro-survival compounds have been reported. A large throughput study by Xu *et al* (2010) found only 2 compounds from a screen of over 50,000 that had a pro-survival effect on hPSC. The first of these compounds, Thiazovivin was subsequently shown to have significant inhibitory effect on ROCK when used at concentrations as low as 2μM. The second of the highlighted compounds, Tyrintegin, did not inhibit ROCK as assessed by an *in vitro* kinase assay but the authors did not investigate any alternative impact on the ROCK signalling pathway such as MLC phosphorylation. They reported that the use of a Pi3K inhibitor completely ablated the protective effect of Tyrintegin (Xu *et al*, 2010) and concluded that this pathway has a role in hPSC survival.

A further study, performed by Barbaric *et al* (2010) demonstrated that pinacidil and HA-1077, as well as 3 other compounds also had a pro-survival effect on hPSC, but again each of these were subsequently shown to be inhibitors of ROCK. Thus, despite employing these high-throughput strategies, a suitable hPSC survival compound that does not inhibit ROCK has not yet been identified (Xu *et al*, 2010; Barbaric *et al*, 2010) and no other mechanism mediating for stem cell death/survival during dissociation has been elucidated.

Our lab recently performed a high throughout assay screening over 20,000 compounds for any positive effect on hPSC survival. This published work (Andrews *et* al, 2010) identified a further 18 survival compounds that were able to promote survival of dissociated hPSC. As with previous work, *in vitro* kinase assays were performed and the results revealed that although some of the compounds were more selective, each of the identified molecules were also inhibitors of ROCK. However, this project identified an additional 19th compound that had no inhibitory effect on ROCK. Further characterisation of this compound, referred to as T16, was not within the scope of that project and it was therefore omitted from the resulting publication.

This thesis describes the characterisation of T16, a novel pluripotent stem cell survival compound that did not inhibit ROCK in an *in vitro* kinase assay, and which will allow not only further investigation in to the pathways involved in dissociation induced cell death but may also provide a platform to accelerate the translation of hPSC derived therapies and products.

The experiments described in chapter 3 of this thesis aimed to further characterise the survival benefits of T16 and to determine if T16 could be used as an alternative to the commonly used Y27632.

The first step taken was to optimise the performance of T16. Although there were significant increases in cell survival when used between 10μM and 40μM, T16 was shown to be most effective at 30μM. A concentration of 50μM resulted in cell survival similar to that of untreated cells, which suggests that at this high concentration T16 may be toxic to hPSC. Pre-treatments ranging from 2hrs to 24hrs resulted in significantly increased cell survival when compared to no pre-treatment, with survival increasing with the length of pre-treatment towards the optimal point at 24hrs. Based on these observations, all subsequent experiments were performed at a concentration of 30μM and with a 24hr pre-treatment. This differs from Y27632 which, in keeping with its known activity as a kinase inhibitor, has been shown to work optimally at 10μM, with a short (1-2hr) pre-treatment followed by use immediately post dissociation (Watanabe *et al*, 2007). Y27632 has also been used to successfully support hPSC survival when added after dissociation but with no pre-treatment (Singh *et al*, 2010). The requirement for pre-treatment as well as the maximum effect at 24hr pre-treatment is perhaps indicative that T16 does not function via direct kinase inhibition (at least as an ATP competitive inhibitor), which is supported by the *in vitro* kinase assay which showed T16 had no inhibitory effect on 120 of the 121 kinases assayed and only a minor effect on RIPK2.

In order for T16 to be accepted as a valid alternative to Y27632, it would be necessary to demonstrate a pro-survival effect comparable to that of Y27632. The body of data generated and presented in chapter 3 shows that T16 treatment not only significantly increased cell survival compared to untreated control cells, but also resulted in cell survival equivalent to Y27632 treatment. Furthermore, T16 can be used to support enzymatic passage of hPSC for at least 30 consecutive passages when used transiently at the point of passage or for at least 20 passages when included continuously in culture medium.

It is important to appreciate the inherent variability of hPSC lines as well as the differences between hiPSC and hESC (Allegrucci and Young, 2007). It is therefore reassuring that the response to T16 was consistent over a number of hESC lines

including the original Wisconsin cell lines H1 and H9 (Thomson *et al*, 1998) as well as a GMP grade cell line; RC9 (Roslin Cells Ltd, Edinburgh). Additionally, the pro-survival effect was observed when a hiPSC (NMF-iPS6) line reprogrammed by Sullivan and colleagues, using retroviruses containing c-Myc, Klf4, Sox2 and Oct4, was used (Sullivan *et al*, 2010).

Similarly, the ability of T16 to support survival regardless of ECM (FN, VN, CS or Matrigel) or culture medium used (StemPro hESC SFM, mTeSR1 or MEF-CM) suggests that the pro-survival effect observed in response to T16 treatment is not an artefact of cell culture but is in fact a direct response to the molecule.

The effect of prolonged exposure to T16 on the expression of key pluripotency related markers was also examined. SSEA4 and SSEA1 have been used as positive and negative markers of pluripotency respectively in a number of publications such as in Thomson *et al* (1998). Critically, both transient and continuous use of T16 had no effect on the expression of pluripotency marker SSEA4 which had consistent expression on >95% of cells for at least 30 passages (20 for continuous use) as determined by flow cytometry. Furthermore, the proportion of cells expressing the differentiation marker SSEA1 remained at <5% throughout, again determined by flow cytometry. In addition, T16 treated cells retained high expression of the core pluripotency transcription factor Oct4 (Babaie et al, 2007; Boyer *et al*, 2005) and of the pluripotency associated cell-surface marker Tra-1-81, as assessed by immunocytochemistry.

An expanded panel of genes with known roles in regulation of pluripotency and the stem cell state, as well as those expressed in differentiated cell types, were also examined at the transcriptional level via qRT-PCR using the TLDA platform. The resulting data showed that treatment with T16 did not result in differential expression of any of the genes tested after 15 consecutive passages. Expression of differentiation markers such as GATA4, FOXA2 and PAX6 remained consistently low whilst components of the core transcriptional regulators of pluripotency such as Oct4, Nanog and Sox2 remained high. Furthermore, there were no significant differences in expression when compared to Y27632 treated cells. This data is further evidence that T16 is a suitable alternative to Y27632 treatment as it does not alter the transcriptional profile of these critical regulators of stem cell identity.

Whenever a new reagent is used in pluripotent hPSC culture systems, it must be shown that it does not interfere with the multi-lineage differentiation capacity of the cells. When Y27632 was first described, Watanabe *et al* (2007) confirmed the pluripotent potential of hESC by teratoma formation in mice as well as via passive differentiation into multiple lineages. However, the method of passive differentiation alone is now more commonly used (Hasegawa *et al,* 2006; Burton *et al,* 2010) as alternatives that reduce animal use are sought. HPSC treated for multiple consecutive passages either transiently or continuously were able to differentiate towards mesoderm shown by staining for smooth muscle actin (SMA), ectoderm (PAX6) and endoderm (alpha fetoprotein, AFP) thereby confirming that prolonged and continuous exposure to T16 does not negatively impact their capacity for multi-lineage differentiation. Furthermore, treated cells were able to undergo differentiation towards red blood cells (RBC) using a feeder-free suspension based protocol (Mountford lab, patent filed).

Having demonstrated that T16 treatment supports hPSC survival upon enzymatic disaggregation without negatively impacting the expression of pluripotency and differentiation markers at both the protein and mRNA transcript level, it was important to determine if T16 treatment lead to abnormal karyotype. Prolonged culture of hPSC can result in chromosomal abnormalities, often the acquisition of an additional copy of chromosomes 12 or 17 (Maitra *et al*, 2005), and the enzymatic passage of hPSC using reagents such as Y27632 has been shown to result in increased incidence of abnormalities (Mitalipova *et al*, 2005). Karyotypic stability, as assessed by G-banding analysis, confirmed that H1 hESC cells treated with T16 or Y27632 remained karyotypically normal for at least 30 passages when used the molecules were used transiently during passage. Furthermore, hiPSC remained normal for at least 20 consecutive passages when T16 was used continuously as a culture medium supplement. However, hiPSC treated continuously with Y27632 had an additional copy of chromosome 12 in 2 of 20 cells analysed at passage 20 (P20). This is likely to be the result of stress and the resulting adaptation caused by continuous use of Y27632. This is supported by data showing a dip in survival of cells continuously treated with Y27632 between passages 10-12 which subsequently recovered. Also in this set of experiments with hiPSC transient use of Y27632 over multiple passages also resulted in an extra copy of chromosome 12 in 1 of the 20 cells analysed at P20,

which is contrasting to results from the earlier separate experiment shown earlier in chapter 3 in which H1 hESC treated transiently with Y27632 retained normal karyotype at P20. These differences might also reflect differential sensitivity to karotypic instability between hESC and hiPSC. Although the abnormalities observed in response to Y27632 treatment could be a result of tissue culture adaptation (Matalipova *et al,* 2005) rather than a consequence of Y27632 treatment, other studies have warned of an increased incidence of aneuploidy in response to treatment with Y27632 (Riento and Ridley, 2003; Liu *et al*, 2012). As all cells in these two experiments originated from the same karyotypically normal hPSC populations (H1 hESC or hiPSC), and only those treated with Y27632 developed any abnormalities, this data could suggest T16 is better suited than Y27632 at supporting normal expansion of hPSC.

The data garnered during the characterisation phase of this study raises interesting and potentially very useful applications for T16. The culture of hPSC is continually evolving and the development of fully defined, xeno free and scalable culture systems is at the heart of the drive to realise the great hope of clinical applications for hPSC (Chen *et al*, 2011; Ludwig *et al*, 2007). We have shown that T16 supports survival of dissociated hPSC in undefined MEF conditioned medium as well as in more defined commercial culture systems such as Stempro and mTeSR. Currently, all of the available media systems rely on additional treatment with Y27632 if single cell suspensions are required and mechanical or partial dissociation methods, that require a significant degree of skill and experience, are widely used during passage to avoid dissociation induced apoptosis. However the use of partial dissociation methods limits the use of the cells for transfection, cloning and cell sorting applications. T16 was added to Stempro™ medium as a continuous supplement with no detectable adverse effects, which allowed the passage and manipulation of hPSC without the requirement of additional drug treatments. This could reduce the complexity of hPSC culture and may aid the development of even more defined ‘all purpose’ culture media that permit the single cell preparation or passage of healthy hPSC whenever they are needed without pre-treatment.

T16 was initially found to lack kinase inhibitor activity and to confirm the result of the previous kinase screen, was retested with an expanded panel of kinases

(performed at The International Centre for Kinase Profiling, Dundee). These results confirmed that T16 did not have any inhibitory effect on ROCK. However, they did highlight some inhibition of RIPK2 (receptor interacting protein kinase). RIPK2 has been implicated in cytokine responses of cells from both the adaptive and innate immune systems via activation of NFκB. RIPK2 consists of an N-terminal kinase domain, linked to a C-terminal caspase activation and recruitment domain (CARD) via an intermediate region and has been reported to induce apoptosis in immune cell types (Moreira and Zamboni, 2012; Tigno-Aranjuez *et al*, 2013; Navas *et al*, 1999). Given the downstream signalling associated with RIPK2, an inhibitor of IκK (TPCA-1) was used to disrupt activation of NFκB mediated signalling, however, this had no effect on hPSC survival (with or without T16 treatment). Western blot analysis was performed on hPSC to confirm the expression of RIPK2, but despite testing numerous antibodies, no positive signal was detected. As T16 was only a weak inhibitor of RIPK2, and given the poor supply of reagents available to investigate this pathway, we decided not to investigate any further. However, RIPK2 is activated by nucleotide oligomerization domain protein 2 (NOD2) a key mediator of homeostasis in the immunological mucosa, and over-activity of this NOD2/RIPK2 pathway is implicated in aberrant inflammatory responses. The inhibition of RIPK2 is therefore a clinically important target for drug development to treat a variety of auto-immune diseases including Crohn's Disease and sarcoidosis (Tigno-Aranjuez et al, 2010) and future investigations of T16's capacity to very specifically inhibit RIPK2 could be interesting in this regard.

After reaffirming that T16 was not an inhibitor of ROCK by repeating the *in vitro* kinase assay on an expanded panel, we next set out to determine if T16 mediates its effect independently of the well described RhoA/ROCK/MLC axis.

It is widely believed that the ROCK mediated hyperphosphorylation of MLC and the resulting actinmyosin induced contractility is the direct cause of dissociation induced apoptosis of hPSC (Chen *et al*, 2010; Ohgushi *et al*, 2010; Walker *et al*, 2010). Treatment with Y27632 leads to a reduction in levels of phosphorylated (active) MLC and thereby increases the proportion of cells that survive. Interaction of T16 with this signalling pathway either upstream or downstream of

ROCK was investigated and the findings were documented in chapter 4 of this thesis.

To investigate whether T16 had the same mechanistic target as Y27632, western blot analysis was used to evaluate expression levels of pMLC in response to dissociation. Strikingly, T16 did not significantly reduce the phosphorylation of MLC when compared to untreated control cells and at several time points resulted in levels of pMLC higher than that seen in control cells; T16 treated cells also had significantly increased levels of pMLC when compared to Y27632 treated cells. The data resulting from Y27632 and untreated control hPSC were consistent with that observed by other groups (Chen *et al,* 2010; Ohgushi *et al*, 2010) which not only confirmed the reliability of these results, but also gave further support to the documented mechanistic action of Y27632, whilst suggesting an alternative mechanism for T16. This was the first evidence for a non-pMLC dependent mechanism for hPSC survival after dissociation.

It has been shown previously that the small GTPases RhoA and Rac1 have an antagonistic relationship through which activation of one can lead to a decrease in the other (Tang *et al,* 2012). Also, Ohgushi *et al* (2010) have suggested that a RhoA-high/Rac1-low status occurs upon dissociation and that constitutively active Rac1 can rescue hPSC from dissociation induced apoptosis. Although previous results suggest that T16 promotes hPSC survival regardless of the phosphorylation status of MLC, the possibility that upstream interaction with the Rho/ROCK/MLC axis, which perhaps favoured a Rac1-high/RhoA-low status, could not be ruled out. Therefore GTPase activation assays were used to determine the activation status of both RhoA and Rac1 in response to dissociation.

The data generated were in agreement with the findings of Ohgushi *et al* (2010), showing a sudden and significant increase in RhoA activation in untreated cells upon enzymatic disaggregation. However, the data presented within this thesis expanded upon this published data and showed that, despite the recent evidence suggesting a possible feedback mechanism through which ROCK inhibition can decrease RhoA activation in a TIAM1 dependent manner (Tang *et al*, 2012), treatment with Y27632 was unable to prevent the sudden activation of RhoA. Similarly, treatment of hPSC with T16 was also unable to prevent the

sudden activation of RhoA in response to dissociation. These data suggested that T16 does not prevent the hyperphosphorylation of pMLC by targeting proteins upstream from ROCK and further supports the hypothesis that T16 mediates survival of hPSC independent of the RhoA/ROCK/MLC axis.

Although activation of RhoA is undeniably a principle component of apoptosis in dissociated hPSC, its activation does not necessarily result in apoptosis. Experiments in chapter 4 showed that treatment with LPA, a well-documented RhoA activator (Frisca *et al*, 2013), resulted in significantly increased levels of GTP bound RhoA in hPSC. However this chemically induced activation did not negatively impact the survival of cells treated with T16 or Y27632. This is not surprising in Y27632 treated cells given that the mechanistic target, ROCK, is downstream of RhoA and Y27632 is therefore, able to block terminal signalling events despite RhoA activation. However, this provided strong evidence that T16 mediated its pro-survival effect independently of the RhoA/ROCK/MLC axis as the activity of both the upstream regulator (RhoA), and downstream target (MLC), were unaffected by T16 treatment.

When the activation status of Rac1 was investigated, there were no significant differences in the levels of GTP bound Rac1 in response to dissociation of untreated cells or those treated with either small molecule survival compound. Although there were detectible levels of active Rac1 in each of the time points measured, the dissociation of hPSC did not result in significant up or down regulation of Rac1 activity (although there was a trend towards decreased activity after 2hrs). These findings suggested that T16 did not mediate its protective effect by promoting Rac1 activity, and are different to those reported by Ohgushi *et al* (2010), who observed a decrease in active Rac1 30minutes post dissociation in untreated cells. This may be due to differences between cell types as the data presented here was generated using hiPSC whereas Ohgushi and colleagues used hESC. However it should be noted that Ohgushi and colleagues did not measure the activation status of Rac1 beyond 30minutes post dissociation and it is unclear if the results reported were reproducible as only a single image is shown. During experimentation, the activation status of Rac1 was highly variable, however, although the data did not reach significance, levels of GTP bound Rac1 did decrease with increasing time post dissociation.

Although this data suggested that T16 did not promote survival via modulation of Rac1 activity, we performed experiments that reaffirmed the critical importance of signalling mediated by Rac1. The use of the Rac1 inhibitor NSC23766 to specifically inhibit the interaction between Rac1 and its associated GEFs, TIAM1 and Trio, resulted in a significant, dose dependent decrease in hPSC survival post dissociation even in the presence of the pro-survival compounds T16 or Y27632. Under normal physiological conditions, integrin dependent cell adhesion is associated with high levels of GTP bound Rac1 which allows the formation of cell protrusions and nascent adhesions which eventually develop into the major signalling complexes known as focal adhesions (Parsons *et al*, 2010; Iden and Collard, 2008; Etienne-Manneville Hall, 2002; Ridley, 2001). The inability to re-establish these cell-to-matrix adhesions clearly has massive implications on the survival of hPSC which cannot be overcome by either of the survival compounds.

A key observation in the response to Y27632 treatment is that in the absence of a suitable ECM or when low attachment culture dishes are used, hPSC form small cell aggregates that not only remain viable (>70%), but under the appropriate conditions will remain pluripotent or can be induced to differentiate (Watanabe *et al*, 2007). In contrast, T16 or untreated cells failed to form cell aggregates when dissociated and placed into suspension culture in standard pluripotency culture medium. Furthermore, within 24hrs these cells in non-adherent culture wells, are largely apoptotic (> 80%) as determined by annexin V/PI assays. This not only highlights a key difference between these two survival compounds but critically shows that survival promoted by T16 was dependent upon cell-matrix adhesion in contrast to Y27632, which can enhance cell-cell adhesion and subsequent survival. However, when NSC23766 was used in combination with Y27632 the hPSC did not re-aggregate and were unable to evade apoptosis. This suggested that the inhibition of ROCK and the subsequent actinmyosin based contractility alone was not sufficient to prevent apoptosis, but rather that it the re-establishment of cell-matrix or cell-cell adhesions that was the critical factor in preventing hPSC from dissociation induced apoptosis.

Although these findings were in disagreement with the dogma that Y27632 acts via inhibition of pMLC, further evidence for the role of cell-cell adhesion has been provided by Krawetz *et al* (2009). They used Y27632 in combination with

the Ca^{2+} chelator EGTA and found that blocking the cadherin based cell-cell adhesion ablated the pro-survival effect of Y27632. Their findings were reproduced when this experiment was repeated within this thesis.

Many groups have linked RhoA activity to the regulation of E-cadherin signalling with conflicting results showing ROCK inhibition increasing or decreasing cell-cell adhesion (Sahai and Marshall, 2002; Harb et al, 2008 and Krawetz *et al*, 2009). Overall, the data described in this thesis suggest that in addition to RhoA, Rac1 may also play a critical role in regulating E-cadherin dependent cell-cell adhesion in hPSC. This raises important questions about the pro-survival mechanism of Y27632 as these results suggest that the re-establishment of cell-cell contact is more important than the inhibition of MLC hyperphosphorylation, and that supporting/triggering this may be the pro-survival mechanism of Y27632.

During this study the morphological appearance of Y27632 and T16 treated hPSC post passage was found to be strikingly different. Whilst T16 treated cells remained well spread and closely resembled untreated control cells, Y27632 treated cells exhibited a characteristic spikey morphology, with numerous spindle-like projections. These morphological differences were further investigated this using the myosin inhibitor Blebbistatin or the RhoA inhibitor C3. HPSC treated with Y27632, C3 or Blebbistatin resulted in this characteristic spikey morphology, reduced cytoskeletal organisation and reduced stress fibres as imaged by both phase microscopy and phalloidin staining of F-actin. This morphology was somewhat expected as a result of C3 or Y27632 treatment given the role of RhoA in actin cytoskeleton organisation (Bristow et al, 2009; Parsons et al, 2010 and Jaffe and Hall, 2005). However, as Blebbistatin does not inhibit ROCK this phenotype was unexpected and suggests that the cause of this phenotype could be the inhibition of actinmyosin induced contractility rather than upstream RhoA signalling. This hypothesis is further supported by data from Chen et al (2010), who observed the same phenotype following RNAi mediated knockdown of MLC and the myosin heavy chain. In contrast, both untreated and T16 treated hPSC resulted in a well spread morphology with well organised, F-actin rich cytoskeleton which further supports the western blot data showing

T16 does not inhibit the hyperphosphorylation of MLC or affect cytoskeletal organisation.

The data presented in chapter 4 indicated that T16 did not have the same mechanistic target as Y27632 (ROCK) and did not appear to interact either upstream (RhoA/Rac1) or downstream (MLC) of the RhoA/ROCK/MLC pathway that has been shown to be critical for Y27632 mediated survival of hPSC. This may make T16 incredibly useful as it will avoid many of the off target effects associated with interference of such a complex signalling pathway

Having concluded that T16 supports survival in a manner independent of the RhoA signalling pathway, the work described in chapter 5 of this thesis focussed on determining the mode of action of T16. Apoptosis assays performed under non-adherent conditions had shown that the pro-survival effect of T16 was dependent upon cell-matrix adhesion. For this reason, investigations into the expression levels of genes with known roles in cell adhesion including integrins, cadherins, and a number of adapter and scaffolding proteins thought to be involved in adhesion dynamics, were performed. Previous groups have reported a critical role for integrins in hPSC maintenance and have shown widespread expression of family members (Meng *et* al, 2010; Rowland *et al*, 2010; Rodin *et al*, 2010 and Miyazaki *et al*, 2012). Using integrin blocking antibodies, Meng and colleagues suggested that the most important integrins for hPSC adhesion were αVß3, α6, ß1, and α2ß1. Analysis of mRNA levels by qRT-PCR confirmed the expression of a number of α and ß integrin sub-units including those above, however, T16 treatment did not significantly alter the expression of any of these integrins at least at the transcriptional level. Flow cytometric analysis of integrin proteins at the cell surface confirmed endogenous expression of a number of these integrins, however, result also showed that T16 did not alter their expression when compared to untreated or Y27632 treated cells. Similar to that seen by Meng and colleagues, >90% of cells were positive for ß1, α2 and α6 and >80% positive for αV when assessed by flow cytometry and the MFI were similar showing that recycling of integrins at the cell surface was unlikely to be affected. Unexpectedly, neither H1 hESC cells nor hiPSC had detectable expression of integrin ß3 as assessed by mRNA or protein level. Analyses in this study were performed with cells cultured on fibronectin, whereas Meng *et al*

used Matrigel. This difference in culture system may account for the difference in expression of integrin ß3 as Matrigel is a more complex undefined product in contrast to fibronectin, and other studies have also reported little or no detectable expression of integrin ß3 in hPSC (Braam *et al,* 2008). Although undoubtedly important, these results suggest that T16 does not directly modulate integrin expression.

As with the analysis of integrin expression, none of the other adhesion related genes analysed were differentially regulated in response to treatment with T16 or Y27632. We therefore targeted intracellular pathways with known roles in adhesion or pro-survival pathways by using kinase inhibitors (Pi3k/Akt, EGFR, Src, PKC and TGFß). The kinase inhibitors were used in the same way as Y27632 in that the cells were given a 2hr pre-treatment with each inhibitor, and the medium was supplemented with each inhibitor post passage and survival was assessed 24hr post dissociation. The results of these studies revealed a role for PKC in survival of hPSC. Inhibition of PKC signalling resulted in increased survival when compared to untreated control cells (2-fold increase in survival) which is consistent with the effect reported by Damoiseaux *et* al (2009) who also observed that inhibitors of PKC increased survival in hPSC. Although inhibition of PKC supported survival, it did not do so as efficiently as either T16 or Y27632. It therefore seemed likely that PKC is part of a complex signalling network which results in hPSC death rather than a direct target of T16, this is supported by the *in vitro* kinase data which showed T16 had no effect on several PKC isoforms included within the panel.

In contrast, the use of the Src-family inhibitor PP2 resulted in very clear difference in the response of T16 and Y27632 treated hPSC. PP2 treatment had no effect on the survival of Y27632 treated hPSC, however when used in combination with T16, PP2 completely ablated the pro-survival effect. This observation was consistent regardless of the cell line used. To further investigate hPSC were treated with PP2 at various time points before and after dissociation and it was clear that the capacity of PP2 to reduce cell survival in a time dependent manner. Untreated hPSC, as well as those treated with T16 or Y27632, had PP2 added either 2hrs pre dissociation (and thereafter), at the point of passage, or 30mins, 1hr, 2hr, 4hr, or 8hr post passage. Addition of PP2

to cultures either 4hr or 8hr post dissociation resulted in a diminished effect of PP2 (~45% and ~60% respectively), with the latter showing no significant difference to T16 alone. However, when used at earlier timepoints post-dissociation, PP2 over-rode T16 mediated survival and resulted in cell death. During routine passage using survival compounds, hPSC begin to adhere to the ECM within the first 1-4 hours following dissociation, therefore, these results could suggest that Src-family kinase activity is required for T16 mediated adhesion to the ECM during this initial time period, but that once cells have re-established contact with the ECM (after 4hours) this activity of Src-kinases is dispensable. Src-family kinases have a well-established role in adhesion signalling and can be activated following integrin activation or downstream of receptor tyrosine kinases (RTK) such EGFR and FGFR (Ray *et al*, 2012; Parsons *et al, 2010* and Moro *et al*, 2002). Once activated Src can not only facilitate the formation of focal adhesions, but can signal downstream of adhesions to promote activation of a number of pathways implicated in cell survival such as Pi3K/Akt and MAPK signalling (Mahajan and Mahajan, 2012; Guo *et al*, 2013; Osaka and Gama, 2013). Src signalling can also interact with components of the RhoA pathway and has been shown to modulate the activation status of RhoA via activation of various GAPs and GEFs such as P190RhoGAP (Papadimitriou et al, 2011; Parsons *et al*, 2010). However, upstream regulation of RhoA is unlikely to be the mode of action responsible for increased cell survival when considering results discussed from chapter 4, which demonstrated that RhoA signalling is not altered by treatment with T16.

Having shown the detrimental effect of PP2 on T16 mediated survival, implicating Src-family kinases in the mode of action of this small molecule, the expression and phosphorylation state of Src-kinases was investigated by western blot using a pan-Src antibody. Src kinases can be phosphorylated (tyrosine) at multiple sites; but, phosphorylation of tyrosine 416 within the kinase domain has been reported to upregulate activity of members of the family (Hunter, 1987). The western blot analysis confirmed that hPSC do express Src-kinases, but surprisingly showed there was no increase in phosphorylation in response to T16 treatment. In fact, although not statistically significant, at many of the critical early time points there was a higher level of phosphorylated Src in the untreated

control cells. This suggests that although the survival mechanism supported by T16 is Src dependent, T16 is not directly regulating Src phosphorylation.

It is important to appreciate that PP2 is a Src-kinase family inhibitor and it remains unclear which specific protein may be causing the effect. The Src-family kinases have extensive structural homology and have been shown to have similar activation loops. As experienced by other groups (Ding *et* al, 2011), this makes the identification of distinct family members difficult. Although both the total Src and pSrc antibodies used in this experiment were directed towards Src, they are reported to also bind other family members including total Fyn, Lyn, Yes and Hck which can also be phosphorylated at the same site (Y416). Transcriptional analysis (qRT-PCR) of a number of these family members confirmed the presence of mRNA for Hck, Fyn, Lyn, Yes as well as Src in hPSC and the use of T16 did not result in any change in transcript levels of any of the family members. Given the non-specific nature of PP2 and that of other reported inhibitors of Src, in order to further investigate which family members may be important in mediating the pro-survival effect of T16, a more interventional approach may be required. Numerous groups have used RNAi as a means through which to further deconvolute signalling pathways which involve Src-family members, and this is a strategy that may be useful for future studies to determine which family members are important in hPSC survival in response to T16 treatment (Taniguchi *et al*, 2013; Iida *et al*, 2013; Tyner *et al*, 2009).

Src family kinases are involved in a plethora of signalling pathways, so in order to further investigate the role of Src-kinases in hPSC survival a higher throughput approach was taken. Using Proteome Profiler™ Antibody Array kits, the activation state of 45 common protein kinases and a further 26 kinases more specifically related to MAPK signalling, were assessed in control and T16, T16 + PP2 or Y27632 treated cells. Expression profiles of interest were predicted to show that PP2 either reduced phosphorylation of kinases that are active in response to T16 treatment or increase activity of those that are not. The data produced by the proteome profilers was problematic to analyse as it is based on mean pixel density of duplicate spots from a single experiment, making it difficult to identify any obvious targets. However, it is reassuring to see that PP2 treatment resulted in a decrease in phosphorylation of Src-family kinases as well

as many downstream Src targets such as FAK, EGFR, PDGFR and Akt. Also, given that T16 was not seen to inhibit kinases in an *in vitro* assay, it is reassuring, if not surprising, that there were no gross changes in response to T16 treatment.

Based on the observation that there was no significant differential regulation of Src-kinase phosphorylation by T16, we began to consider the possibility that T16 may alter the relationship between Src-kinases and their upstream or downstream targets and/or alter sub-cellular localisation of Src-kinases. Receptor for activated protein kinase C (RACK1) was the most highly expressed adhesion-related factor identified during our custom adhesion array (qRT-PCR) and was confirmed to be expressed at the protein level in both hESC and hiPSC by western blotting. RACK1 is a very well conserved scaffold protein thought to be expressed in all mammalian cells and was original discovered as a binding partner for PKC (isoform βII). However, it has recently been shown to be involved more widely in signal transduction pathways either directly or by modulation of its binding partners (Adams *et al*, 2011; Ron *et al*, 2013; Cox *et al*, 2003; Sutton *et al*, 2013). RACK1 has also been implicated in the regulation of cell adhesion, morphology and migration mainly via its interactions with Src-family kinases and integrins (Doan and Huttenlocher, 2007; Mamidipudi and Cartwright, 2009; Kiely *et al*, 2005; Trerotola *et al*, 2012; Levesque *et al*, 2006). Mamidipudi and Cartwright (2009) have suggested that RACK1 is able to inhibit Src activity by binding to it, this subsequently reduces the activation of the anti-apoptotic Pi3K/Akt signalling pathway. The role of cell-matrix adhesions, mediated primarily by integrins, has been heavily associated with not only the regulation of pluripotency in hPSC, but also in the activation and suppression of various signalling pathways triggered by dissociation (Ohgushi *et al*, 2010; Watanabe *et al*, 2007; Krawetz *et al*, 2009; Meng *et al*, 2010). Therefore, it is possible that RACK1 is a mediator of these critical pathways in hPSC, possibly via binding to and reducing the activity of Src-kinases, and that T16 may alter this interaction and thereby increase stem cell survival. Regulation of this type is not uncommon in cells and has been alluded to earlier when discussing the roles of GDIs in regulation of small GTPases, wherein GDIs bind GTP bound RhoA and alter its sub-cellular localisation, thereby preventing it from binding to (and activating) its targets (Garcia-Mata *et al*, 2011). Furthermore, in a comparative transcriptome analyses comparing pluripotent cells from human and mice to that

of planarians, Labbe *et* al (2012) found that RACK1 was one of a small set of genes shown to be critical to the maintenance of all three pluripotent cell types (Labbe *et* al, 2012). RACK1, therefore, was a promising candidate factor in the mechanism by which Src-kinases modulate T16 mediated survival.

To investigate this further we used a GST RACK1 fusion protein as well as immunoprecipitations with and without T16 treatment. We performed co-immunoprecipitations using RACK1 to pull-down Src-kinases and found that treatment with T16 resulted in a significantly reduced level of Src-kinases bound to endogenous RACK1 within hPSC when compared to the untreated cells. Furthermore, when the GST RACK1 fusion protein was incubated with hPSC lysates, pulldowns from cells treated with T16 also showed a decrease in precipitated total Src-kinases when compared to both the Y27632 treated and untreated cells.

The identification of RACK1 as a potentially important mediator of apoptosis in dissociated hPSC may also partly explain why we and others have found PKC inhibition to have beneficial effect on hPSC survival (Damoiseaux *et* al, 2009). It has been documented that the activation of PKC results in the co-localisation and subsequent binding of Src and RACK1 (Chang *et al*, 2002). Inhibition of PKC may therefore lead to reduced binding between Src and RACK1 which would leave Src free to bind alternative intracellular substrates such as those promoting adhesion to the ECM such as integrins or FAK.

The identification of a member, or members, of the Src-family kinases as important regulators of apoptosis in hPSC highlights a novel and alternative pathway which may further enhance the current understanding of dissociation induced apoptosis of hPSC. Src-family kinase signalling has been relatively poorly studied in hPSC and to the best of our knowledge has not been directly linked to apoptosis/survival in these cells. A number of groups have however implicated Src as a mediator of differentiation in hPSC. Using a Src-family inhibitor (SU6656), Lian *et al* (2013) were able to improve directed differentiation towards epithelial cells. This group observed increased expression of endothelial markers cytokeratin 8 and cytokeratin 18, which they attributed to altered sub-cellular localisation of ß-catenin in response to Src inhibition (Lian *et al*, 2013). Furthermore, treatment with PP2 was found to enhance differentiation toward

ß-cells as measured by increased expression of transcription factors PAX4 and NEUROD1. The authors proposed that loss of Src activity led to a decrease in FAK activation which subsequently decreased activation of TGFß mediated signalling pathways (Afrikanova *et al*, 2011).

Take together these data highlight a potential and intriguing role for RACK1 as an *in vivo* regulator of Src-family activity and suggest that this relationship may be altered in response to T16 treatment. These data suggest that there is less intracellular Src bound to endogenous RACK1 or pulled down by GST RACK1 in cells treated with T16, and that a Src-kinase may therefore have altered sub-cellular localisation in response to T16. This merits further investigation which could include co-IP of additional Src-kinases binding partners such as integrin ß1 or FAK. It is possible that T16 treatment results in the release of Src from RACK1, facilitating the interaction of Src-kinases with integrins, paxillin or FAK, which allows hPSC to more efficiently form the critical cell-matrix adhesions that support survival. Alternatively Src-kinases may activate pathways critical for protection of hPSC from apoptosis such as PI3K/Akt or EGFR signalling which could allow hPSC to survive for long enough to re-establish 'normal' signalling via cell-matrix or cell-cell interactions.

The process of anoikis has in the past been associated with the dissociation induced apoptosis of hPSC (Watanabe *et al*, 2007; Krawetz *et al,* 2009). Anoikis is a specialised form of apoptosis triggered by loss of cell to matrix adherence and many proteins, including those shown within this thesis to be critical to T16 mediated survival such as integrins and Src-kinases, have been heavily linked to anoikis. Work performed by Haenssen *et al* (2010) showed that both integrin α5 and Src, which have been confirmed to be expressed in hPSC, were indispensable in the development of anchorage-independence in mammary epithelial cells. That work also suggested that the two act together to activate several anti-apoptotic pathways via suppression of the pro-apoptotic Bim, which subsequently protects the cells from anoikis. Other groups have suggested that ß1 integrin, Src and FAK are key orchestrators of both pro-apoptotic and anti-apoptotic signalling in response to adhesion, or loss of, through regulation of multiple Bcl-2 family members and downstream signalling mediated by Pi3K/Akt and MEK/Erk (Bouchard *et al*, 2008).

Thus, given the well documented role of Src-kinases in resistance to anoikis, it is possible that if treatment with T16 results in less intracellular Src-kinases being bound to RACK1, the available kinase may instead promote resistance to anoikis via interactions with FAK, integrins and anti-apoptotic signalling pathways (Beausejour et al, 2012; Bolos et al, 2010). Given the plethora of pathways that can be directly or indirectly regulated by Src-family activity, a higher throughput proteomic or metabolomics approach may be required in order to further investigate the role of Src-kinases in T16 mediated survival that has been identified in this thesis.

As T16 is not a kinase inhibitor and significant pre-treatment time (>4hrs) enhanced the pro-survival effect, it was possible that it was affecting transcriptional modulation rather than post-translational modification of signalling components. In an effort to better understand the transcriptional response to dissociation, and also to determine if there was any differential expression in response to treatment with T16 or Y27632, an Affymetrix based microarray experiment was designed and performed using hiPSC. To our knowledge, no previous publications have investigated the complete transcriptional response to dissociation in hPSC.

To ensure results were as reliable as possible, the hPSC to be used were tested for normal karyotype and expression of pluripotency markers such as SSEA4 prior to use in the assay. Furthermore, to maximise the chance of observing changes, apoptosis assays were performed to confirm the differences in phenotype at the time points chosen for analysis. The results showed that 8hr post dissociation, T16 and Y27632 treated cells were largely negative for the marker of apoptosis annexin V, whereas untreated cells were mostly positive. This was consistent with that seen by other groups who also observed expression of apoptotic markers at this time point (Ohgushi *et al*, 2010).

One of the aims of this study was to determine if T16 treatment transcriptionally primed hPSC for survival post dissociation. However when the results were analysed and compared, there was very little differential expression between surviving T16 treated cells compared to the Y27632 or untreated samples which suggested this was not the case. This pattern was similar when making comparisons between samples collected immediately before or following

dissociation. The lack of difference between any of the 3 treatment types at this point was somewhat surprising given that Rho GTPases are well known to influence gene expression (Evelyn *et al*, 2007; Bell *et al*, 2013). This data does however suggest that T16 does not interfere with normal transcriptional regulation even though it is present within the culture medium for 24hr prior to disaggregation.

A very clear observation made whilst analysing the complete data set was that although there were several comparison groups with >1000 differential expressed probes (see table 6.1), the scale of the changes was very small. The vast majority of statistically significant changes were changed by <2 fold. Although a number of these changes were in genes with roles in adhesion or apoptosis and may therefore be biologically relevant, the size of the fold changes makes the validation of target genes more difficult.

The comparison between the cells collected 8hr post-dissociation and those collected immediately following dissociation produced the most differentially expressed genes and was therefore the focus of all subsequent comparisons. As cells at this stage are going through the processes of adhesion and migration, or apoptosis in the case of untreated cells, it would be expected that a large set of these changes would have been observed in genes relating to these processes. The data sets were uploaded to the IPA software which generates a list of canonical pathways, functions and upstream regulators predicted to be altered in response to each data set. We initially focussed on the top 20 canonical pathways and observed that although there were similar pathways altered in response to each treatment type, there were some key differences. Amongst these were the Pi3K/Akt and integrin signalling pathways, which were both in the top 5 pathways impacted in response to T16 and Y27632 treatment, but not in the top 20 of untreated cells. The regulation of the integrin signalling pathway was interesting, but not altogether surprising given that the cells are actively re-attaching and migrating on the ECM following dissociation. Furthermore, previous data had shown a critical role for integrins in the survival mediated by T16 (figure 4.11).

The regulation of the Pi3K/Akt pathway was also interesting as previous results have confirmed the critical importance of signalling mediated by Pi3K. When the

Pi3K inhibitor PI-828 was used, there was a significant decrease in survival mediated by both T16 and Y27632 (figure 5.8). Furthermore, previous groups have shown that Pi3K can be activated downstream of Src activation and that this can subsequently lead to decreased apoptosis (Bouchard *et al*, 2008; Guo *et al*, 2013). Additionally, Ohgushi *et al* (2010) highlighted Pi3K as a potential salvage pathway able to rescue hPSC downstream of the hyperphosphorylation of MLC. The transcriptional regulation of this pathway in response to dissociation is further evidence of the critical role that the Pi3K/Akt pathway pathways plays in the maintenance of hPSC and their response to dissociation (Eiselleova *et al*, 2009; Ohgushi *et al*, 2010; Wang *et al*, 2009).

We chose to validate (by qRT-PCR) a number of components of these pathways including several integrin sub-units, FAK, paxillin and Pi3K isoforms. These were validated using the original RNA used to perform the microarray. The validation results showed that although there were significant increases in expression of these genes between the time points, there are no significant differences between the three treatment groups. This suggests that although these pathways are highly regulated upon dissociation, the responses are not specific to treatment type, which was surprising. Given that the majority of untreated cells are in suspension and undergoing apoptosis at the 8hr time point, whereas T16 and Y27632 treated cells are largely adherent, it would be expected that expression of adhesion related genes would be different. This may suggest that the effect mediated by the survival compounds is largely post translational rather than transcriptional. In addition to individual pathways, IPA predicted cellular functions that were affected by dissociation and survival compound treatments. IPA predicted 101 functions that were expected to have increased activity and 16 predicted to have decreased activity. Approximately 20% of the functions predicted to be increased were shared between the three treatment types, including increased cell survival. This suggests that although treatment with T16 and Y27632 results in vastly different outcomes when compared to untreated cells, the transcriptional response in many pathways is very similar.

Of the relatively few functions predicted to be decreased, 5 of these were shared by T16 and Y27632 but not untreated cells, all of which were related to apoptosis, including cell death and apoptosis of embryonic cells. As might have

been expected, anoikis was one the function that was predicted to be decreased in response to T16 treatment. As discussed previously the pathways involved in resistance to anoikis may be potential targets of T16, particularly those involving Src-family kinases (Beausejour et al, 2012; Bolos et al, 2010). Surprisingly, anoikis was also predicted to be decreased in the untreated cells, but not the Y27632 treated cells. This could suggest that the transcriptional changes were significant enough to be detected from the relatively small population of untreated cells that go on to survive. This could however be further evidence that the transcriptional response to apoptosis has similarities regardless of treatment type.

We also investigated individual gene changes associated with the response to dissociation and found that 26% of these were shared regardless of treatment type and that a further 30% of these were shared between T16 and untreated cells. This again shows a surprisingly similar transcriptional response between the treatment groups. However there were 1008 gene changes associated with T16 treatment alone. We therefore chose to validate those that were associated with relevant cellular processes such as adhesion or apoptosis, including components of FGF, EGF, MAPK and integrin signalling pathways. As before, although a number of these targets resulted in increased expression following dissociation, they did not result in any significant differences between the three treatment groups during validation. The failure to validate targets may reflect the relatively small fold changes that were detected during the arrays.

Taken together, the data garnered during the microarray analysis performed in this chapter further highlights the critical role that the Pi3K/Akt signalling pathway plays in the regulation of hPSC. The Pi3k/Akt pathway was one of the most highly regulated pathways in response to dissociation and treatment with either Y27632 or T16. The data also confirms the importance of integrin mediated signalling in response to dissociation, with many integrin sub-units being up-regulated in the generated data sets. The transcriptional response to dissociation was more similar between treatments than expected, however, it has been reported in the literature that apoptosis can proceed even in the absence of the nucleus (Schulze-Osthoff et al, 1994). This may suggest that the activation of apoptosis in untreated hPSC occurs mainly at the post-translational

level such as through the cleavage of proteins by caspases (Cohen, 1997). Overall, the data garnered from this array analysis suggests that it is unlikely that T16 mediates its pro-survival effect at the transcriptional level.

The data generated within this thesis highlighted a novel Src-kinases dependent, RhoA independent pathway through which hPSC can mediate survival in response to dissociation. However it also creates a number of interesting questions that given more time would have been investigated. The use of the Src-family inhibitor PP2 initially demonstrated the critical role for Src-kinases, however this does not give any indication as to which family member (or members) are playing the critical role within hPSC. Therefore, an RNAi based investigation could be carried out to selectively knock down each family member to determine which are essential to T16 mediated survival of hPSC.

Similarly, given the variety of Src-kinases that were found to be expressed in hPSC, pan-Src family antibodies were used give the best chance of detecting any association and change in binding to RACK1, however this approach precluded the identification of the specific Src-kinase, or kinases. Naturally, additional studies to specifically identify and modulate the Src-family kinase(s) that are important in this system would have been undertaken if time had permitted and are a priority for any future studies.

The data from co-IP studies also supported a critical role for RACK1 in regulating intracellular Src-kinase activity, this relationship could also be further investigated. To do this, a RACK1 mutant with a Y246F mutation that prevents the binding of Src-kinases could be used. This would require the use of viral vectors to transduce cells with the mutant protein and would require substantial optimisation. It would be expected that such a mutation would produce a similar result to that of T16 treatment.

Intriguingly the data from pull down assays clearly demonstrated that whilst the total amount of Src-kinase protein was not altered by T16, the amount pulled-down by RACK1 was significantly lower after T16 treatment. This finding suggested that the intracellular localisation and/or the binding of Src-kinases to other partners may be altered by T16. Given the well documented relationship between Src-kinases and integrins or FAK in the literature, as well as the

adhesion-dependent nature of survival mediated by T16, these would be the logical targets for further investigation. As performed with RACK1, co-IP assays could be undertaken using FAK or integrin β1. Additionally, confocal microscopy or FRET analysis could be used to investigate the sub-cellular localisation and compartmentalisation of Src-kinases in response to T16.

Although we have identified pathways that are critical in T16 mediated survival, the direct target of T16 has not yet been determined. For example it remains unclear whether T16 binds to RACK1 and effectively blocks the interaction of RACK1 and Src-kinases directly, or whether T16 targets upstream of RACK1 and indirectly reduces the capacity of the kinase to bind to RACK1. To address this issue, techniques such as that used by Ong *et al* (2009) could be employed. Here they coupled their identified small molecule to beads in order to identify binding partners, which they subsequently identified using mass spectrometry.

As well as facilitating the survival of hPSC post dissociation, Y27632 has been reported to improve recovery from cryopreservation and to aid the recovery from hPSC following FACS. In addition, Y27632 has also been used during the reprogramming of somatic cell types towards pluripotency. At present we are unaware of the effects that T16 will have on such processes. It may be possible that T16 further improves the recovery of cells following cryopreservation, or may even be a useful small molecule that can further improve the reprograming efficiency and safety of hiPSC. Extending the possible uses of T16 in this way would make it an even more attractive molecule for commercialisation and widespread use.

The structure of T16 was not disclosed during these studies, however the compound was identified from a screen of commercially available drug-like moieties and can be identified for future studies. Knowledge of the structure would allow a medicinal chemistry project to produce structural analogues of T16 and to test their capacity to promote hPSC survival. In this way a basic structure-activity relationship could be identified and may also be possible to produce a more potent analogue of T16. It is also probable that identification of the structural components of T16 that are responsible for its activity would help direct experimental design and give further insight into the biological mechanism of action of this important molecule. Furthermore, given the

potential commercial value of T16, developing the structure activity relationship will be critical in producing sufficient information to protect the structure and use of T16 by filing patent applications for the use of a series of active compounds, and additionally for intervention in any pathway identified. The importance of this commercial potential for T16 has precluded publication of the findings described in this thesis until such patent protection has been secured.

HPSC have vast potential for a number of applications such as in drug discovery and as a source of replacement cells and tissues for use in regenerative medicine. Successful exploitation of this potential will ultimately require mechanised expansion of these cells in order to produce clinically relevant cell numbers. The most straighforward means of acheiving this scale up will involve the enzymatic passage of hPSC but, as discussed in this thesis, hPSC are incredibly sensitive to dissociation. It is therefore clear that compounds that promote survival post dissociation will play a critical role in facilitating the transition from small scale manual culture to large scale or mechanised culture systems. Currently, the inhibition of ROCK mediated signalling using Y27632 or alternative ROCK inhibitors remains the only available option to support survival upon enzymatic disaggregation of hPSC. However, as described previously, there are a number of drawbacks associated with the use of Y27632, as exemplified by the negative effect on cell proliferation and detrimental effect on the production of haematopoietic precursor cells (Joo *et* al, 2012; Yung *et al*, 2011; Zweigerdt *et al*, 2011; Singh *et al*, 2012). T16 therefore has huge potential as an alternative to Y27632 during the development of scalable culture systems.

Suspension culture systems have recently become attractive options for the scale-up of hPSC, as culture without ECM components is inherrently less variable and also more cost effective (Wang *et al*, 2013; Chen *et al*, 2012; Larijani *et al*, 2011). Although T16 does not facilitate the survival of hPSC in suspension culture, this issue is not insurmountable as it is probable that capacity of T16 to promote cell-matrix rather than cell-cell adhesions could be exploited to mediate the survival of hPSC by using ECM-coated bead/carrier based suspension culture systems which could also allow differentiation in the same vessels.

Although the potential use of T16 as a reagent for large scale culture of hPSC is commercially very appealing, its use at small scale must not be overlooked. The

drive towards fully defined and GMP compliant culture media is critical to the successful exploitation of hPSC. The most promising example of this currently available is Essential 8 (Chen *et al*, 2011), which is entirely serum and xeno-free. However, Essential 8 relies on the use of partial dissociation methods using EGTA during passage. Such passage methods are heavily dependent upon the skill of each individual user, as even slight overexposure to EGTA will result in complete dissociation which will cause apoptosis of hPSC. At the time of development (2011) Y27632 was widely available, but the authors interestingly chose to omit it from the formulation of Essential8, possibly due to the adverse effects caused by continuous exposure to Y27632. As to the best of our knowledge, T16 does not result in these issues, it would be a prime candidate for inclusion within culture mediums such as Essential 8 as it considerably simplifies the culture of hPSC and permits manipulation of the cells whenever it is desired without the need for pre-treatment.

7.1 Conclusion

The work undertaken during this thesis aimed to characterise the effect of a novel pluripotent stem cell survival compound known as T16. The data presented within provide evidence that T16 has a previously unreported mode of action and, unlike other survival compounds described to date, does not mediate its pro-survival effect via ROCK inhibition or by interaction with other components of the RhoA/ROCK/MLC pathway. T16 not only supports long term culture of hPSC when used either transiently or continuously, but does so with no observed detrimental effect on the stem cell identity or function, with treated cells expressing high levels of pluripotency markers, maintaining normal karyotype and retaining multi-lineage differentiation capacity.

Taken together, the investigations described within this thesis have identified a previously undescribed ROCK/pMLC independent, Src-kinase dependent mechanism of hPSC cell survival that can be manipulated to avoid dissociation induced cell death. The scaffold protein RACK1 has been identified as an intracellular binding partner of Src-kinases in hPSC and may play a critical role in the regulation of Src-kinase activity by modifying its sub-cellular localisation. Importantly, this newly identified pathway can be mediated by T16 resulting in hPSC survival after dissociation to single cells which is also dependent upon the

reattachment of cells to the ECM. This pathway can be further interrogated and elucidated using T16 and related compounds.

The identification of T16 as an alternative survival compound, that can be used without the detrimental effects observed with Y27632, imparts significant scientific and commercial value to this molecule which will also be an important tool to improve stem cell media, culture and manipulation and may thereby accelerate the drive towards the use of hPSC derived therapies as a viable clinical option.

Appendix

Table shows the $2^{\Delta Ct}$ x1000 values of the entire list of genes analysed during experimentation performed in section 3.3.4. The data showed no differences in gene expression between drug treatment and passage number. Data shown as N/A had either an undetermined $2^{\Delta Ct}$ x1000 value or a negligible value of <0.1.

		Y27632 ($2^{\Delta Ct}$*1000)				T16 (2ΔCt*1000)			
Gene Description	**Gene**	**P0**	**P10**	**P15**	**P5**	**P0**	**P10**	**P15**	**P5**
Core pluripotency transcription factors	**NANOG**	136.5	143.1	128.0	168.8	174.9	209.6	118.9	140.7
	POU5F1	4.1	2.7	2.7	4.1	4.5	4.3	3.4	2.9
	SOX2	53.0	35.0	32.8	50.9	50.0	50.2	40.3	47.5
Expressed in undifferentiated cells	**TDGF1**	113.0	103.1	96.9	118.3	118.4	100.9	78.8	95.4
	DNMT3B	381.0	598.2	526.2	510.5	490.5	629.0	581.5	419.8
	GABRB3	53.5	60.7	53.7	63.3	63.8	83.2	53.9	52.8
	GDF3	2.3	2.0	2.5	3.4	2.2	2.4	2.1	2.8
Implicated in control of stemness	**BRIX**	42.2	35.7	34.2	36.4	49.7	39.9	31.7	36.9
	CD9	83.6	102.1	114.9	110.7	96.6	118.3	99.8	91.1
	COMMD3	0.7	0.8	0.8	0.9	0.7	1.0	0.8	0.8
	CRABP2	151.3	247.9	141.4	142.8	163.4	164.6	251.0	147.3
	EBAF	5.7	2.2	0.6	1.2	11.4	1.8	1.3	3.2
	FGF4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	FGF5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	FOXD3	29.0	28.3	14.0	15.9	34.0	25.7	12.7	13.4
	GAL	55.9	51.8	35.7	85.5	59.4	87.7	30.6	41.4
	GBX2	0.6	0.7	0.2	0.1	0.5	2.5	0.1	0.4
	GRB7	23.7	39.6	28.0	28.8	26.1	30.4	27.7	24.0
	IFITM1	97.7	120.9	87.9	151.4	104.1	118.6	67.5	93.3
	IFITM2	55.4	64.9	59.8	81.2	63.6	70.5	43.9	57.2
	IL6ST	2.4	2.3	2.3	2.9	3.3	2.7	2.5	2.9
	IMP2	13.4	16.9	11.2	15.6	16.4	18.0	12.9	16.4
	KIT	5.7	6.2	5.1	5.4	7.5	5.8	5.1	3.9
	LEFTB	11.0	4.3	3.3	5.0	24.4	6.2	4.5	7.8
	LIFR	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	LIN28	412.9	419.2	337.3	399.5	505.2	450.9	360.6	401.6
	NODAL	39.3	34.2	31.4	47.8	65.5	62.4	22.6	44.5
	NOG	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	NR5A2	3.6	3.9	3.1	3.5	6.0	7.5	2.4	3.6
	NR6A1	48.7	50.0	43.8	49.1	51.2	62.4	46.7	48.8
	PODXL	322.2	467.0	341.5	323.0	389.1	486.9	403.9	388.3
	PTEN	1.3	1.4	1.0	1.7	1.6	1.3	1.3	1.5
	REST	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	SEMA3A	17.8	16.5	20.4	24.9	23.3	23.4	15.2	19.8
	SFRP2	126.2	128.6	102.5	108.5	142.2	156.7	76.7	129.4

	TERT	3.7	3.9	3.0	3.4	4.3	4.3	3.4	3.2
	TFCP2L1	1.1	1.3	0.8	0.9	1.6	1.8	0.8	0.9
	UTF1	2.2	2.8	1.9	1.9	2.6	3.3	1.9	2.7
	XIST	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	ZFP42	104.7	124.5	94.1	92.3	108.5	145.3	101.7	92.6
Differentiation marker	**ACTC**	10.8	7.0	4.8	7.3	12.5	5.9	5.8	6.3
	AFP	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	CD34	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	CDH5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	CDX2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	CGB	0.4	0.5	0.3	0.4	0.4	0.4	0.3	0.3
	COL1A1	95.0	157.7	150.2	114.1	117.0	176.7	199.5	155.2
	COL2A1	3.5	5.9	5.1	4.8	5.0	8.2	4.5	3.3
	DDX4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	DES	2.9	5.6	4.2	5.0	3.8	5.1	5.0	7.7
	EOMES	5.6	4.3	1.2	3.8	5.0	4.1	1.7	4.8
	FLT1	6.1	8.4	6.7	7.2	6.2	7.3	7.5	5.8
	FN1	189.9	55.0	30.6	90.2	209.1	66.5	44.5	34.7
	FOXA2	0.7	0.4	0.1	0.5	0.8	0.4	0.6	0.4
	GATA4	1.6	0.2	0.1	0.9	1.8	0.2	0.6	0.1
	GATA6	2.0	0.1	0.1	2.9	2.0	0.2	0.4	0.2
	GCG	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	GCM1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	GFAP	0.3	0.2	0.2	0.2	0.3	0.3	0.2	0.2
	HBB	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	HBZ	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	HLXB9	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	IAPP	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	INS	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	IPF1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	ISL1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	KRT1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	LAMA1	13.6	13.1	11.8	13.8	15.2	13.5	12.9	11.7
	LAMB1	11.3	13.5	11.2	13.9	13.6	17.5	14.3	11.8
	LAMC1	27.4	25.2	25.1	26.0	35.3	30.1	30.8	23.4
	MYF5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	MYOD1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

	NES	31.7	48.9	36.2	47.6	46.4	70.3	50.4	47.1
	NEUROD1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	NPPA	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	OLIG2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	PAX4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	PAX6	0.4	0.5	0.5	0.4	0.4	0.5	0.2	0.1
	PECAM1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	PTF1A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	RUNX2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	SERPINA1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	SOX17	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	SST	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	SYCP3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	SYP	1.6	2.6	1.5	1.7	2.1	2.4	2.4	1.7
	T	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	TAT	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	TH	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	WT1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table A1- Summary of all 2ΔCt x1000 values from analysis of pluripotency related genes (Section 3.3.4)

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